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Cultural and biochemical characterization, serotyping and pathogenicity of *Campylobacter fetus* subspecies *jejuni*/*Campylobacter coli* of human and animal origin

by

AbdelRhman Bushara SultanDosa

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GENERAL INTRODUCTION

Preamble

Campylobacters, formerly "Vibrios," are gram-negative curved bacteria that occur principally in the gut, and the oral cavity of man and animals and occasionally in the genital tract of animals. They are distinguished from members of the genus Vibrio by their microaerophilic, nonfermentative, thermophilic characteristics and by their percentage of guanine and cytosine (% G&C) content. These differences led to their removal from the genus Vibrio and the creation of the new genus Campylobacter (67). The frequent isolation of campylobacters from various animal species and man in recent years has demonstrated that these organisms are more widespread in nature than was formerly believed.

The catalase positive members of this genus cause infectious infertility in cattle, abortion in sheep and cattle, diarrhea and dysentery in various animals, and are associated with several disease conditions in man, especially enteritis. In addition, some of the catalase positive campylobacters appear to be less pathogenic, commensals and/or symbionts in the intestinal tract and have been isolated from feces of healthy animals, poultry and man (18, 74).

The taxonomic position of the new genus was revised (73, 85), but unfortunately two different classification schemes emerged. This situation undoubtedly reflects our lack of complete information about the exact biochemical reactions and serological relationship of these organisms. In this dissertation, I will sustain the use of the terminology provided by Smibert (73) in Bergey's Manual of Determinative

Bacteriology for the genus Campylobacter. According to this classification, the catalase positive campylobacters comprise 3 species: "Campylobacter fetus, Campylobacter coli and Campylobacter fecalis," and the 3 subspecies "jejuni, fetus and intestinalis" constitute the first species. But the differentiation of Campylobacter species that was based on a limited number of tests is being challenged by more recent studies (48, 70), and dissatisfaction with the current classification is being expressed. As a result, C. fetus ssp. jejuni and C. coli as a group of closely related organisms is being referred to as the "jejuni/ coli group," although sufficient evidence has not been accumulated to classify them into one species.

Campylobacteriosis is now recognized as a zoonosis. As our knowledge of the epidemiology of the disease is quite superficial, there is an urgent need for serotyping the ever increasing number of jejuni/ coli isolates obtainable from human and animal sources. Development of serotyping schemes and determination of the exact antigenic relationship between the various isolates will contribute to a better understanding of the public health significance of these organisms. Also, the members of the jejuni/coli group seem to include pathogenic and nonpathogenic strains. How do we differentiate the pathogenic strains from the nonpathogenic and which strains from the various hosts are the most pathogenic? What biological models are suitable for determining the pathogenic effect of strains? These are some of the questions which need answers to aid in studying the pathogenic mechanisms of Campylobacter.

Objectives

The objectives of the present studies were: i) to investigate the cultural and biochemical characteristics of C. fetus ssp. jejuni/C. coli strains in order to organize them into distinct groups, ii) to serotype C. fetus ssp. jejuni/C. coli strains isolated from man and six animal species in order to determine their antigenic relationship to preestablished serotype strains, iii) to determine the abortifacient effect of C. fetus ssp. jejuni/C. coli strains in pregnant guinea pigs in order to establish their pathogenicity.

Thesis/Dissertation Format

This dissertation is presented in alternate format including three manuscripts to be submitted to scientific journals for publication. Two manuscripts will be submitted to Journal of Clinical Microbiology and one manuscript will be submitted to American Journal of Veterinary Research. All manuscripts in this dissertation are presented in the format of Journal of Clinical Microbiology. A literature review precedes the first manuscript and a general discussion and conclusions follow the final manuscript. Literature cited for each manuscript is listed at the end of the manuscript. Additional literature cited is listed at the end of the dissertation.

The Ph.D. candidate, AbdelRhman B. SultanDosa, was the principal investigator for each of the studies and is the senior author for each of the manuscripts. Other persons with direct but minor involvement in each study co-authored the manuscripts. Specific assistance from others is indicated at the end of the dissertation as an acknowledgment.

LITERATURE REVIEW

The generic name Vibrio has been used for all aerobic, microaerophilic and anaerobic bacterial species of animal and human origin and saprophytic strains isolated from soil and water, as long as they are motile and displayed the basic vibrioid cell morphology. Later it was determined that this is not sufficient criteria. So the microaerophilic vibrios were removed and assigned to the new genus "Campylobacter"[a], as we will see in the following paragraphs.

Historic background: In 1854, Pacini, cited by Wilson and Miles (90), observed motile curved rods in duodenal contents of human patients dying of cholera disease and coined the name Vibrio cholera for these organisms. Isolation of microorganisms that resemble V. cholera in morphology from animals was first reported by Gamaleia in 1888, who recovered them from fowls in Odessa, Russia (26). Later, in 1913 McFadyean and Stockman (52) made the first isolation of Vibrio organisms that morphologically resemble Pacini's Vibrios from aborting sheep. But not until 1918 when Theobald Smith (75) isolated similar organisms from fetuses of aborting cattle did Smith and Taylor (78) name the organism Vibrio fetus (V. fetus). Florent (36) isolated a vibrio-like bacterium from cattle and named it V. bubulus. Then Florent (37) realized that V. fetus can occur in the genital tract or the intestinal tract of cattle

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[a]Any reference to the word Vibrio in this literature review means Campylobacter unless otherwise indicated.

and sheep and infections produced by the organisms isolated from these two different habitats are slightly different. Consequently, he named the Vibrio of enzootic sterility and abortion in cattle Vibrio fetus var. venerealis and the Vibrio of sporadic abortion in cattle and enzootic abortion in ewes Vibrio fetus var. intestinalis. Studies by Jones and Little (43) revealed that microorganisms isolated from the jejunum of cows and calves having intestinal disorders were morphologically similar to V. fetus. Due to the frequent isolation of this organism from the jejunum, Jones et al. (44) named it V. jejuni. Doyle (29) isolated similar organisms from the colonic mucosa of pigs suffering from dysentery and named them V. coli. Vinzent (86) reported the first human case of V. fetus infection. From the blood of a pregnant woman who later aborted, Vinzent et al. (87) isolated and identified the organism as V. fetus. Morphologically similar organisms were also found to infect the gastrointestinal tract of humans by Levy (51), who observed them in stool specimens and called them "vibrio-like." Involvement of Vibrios with hepatitis in chickens was reported by Hofstad (40) and confirmed by Peckham (56).

It is evident from these reports that vibrio or vibrio-like organisms were etiological agents of several diseases in man and animals. However, the trouble arises from the fact that most of these reports depended solely on the morphology and the habitat of the isolates in selecting names for the organisms they isolated. As a result those characteristics of taxonomic importance did not play a major role in early nomenclature of these organisms and that was the basic mistake

which brought about the confusion associated lately with classification of these organisms. There were several problems that faced those early workers. Initially, they were unaware of the microaerophilic nature of the vibrios, a fact that impeded their isolation efforts. Then contamination problems surfaced due to lack of proper isolation media and as characterization and typing techniques were not well developed, there was practically no way for them to establish a sound classification scheme for the various vibrios.

Bacteria are generally classified and grouped into genera and species on the basis of a number of characteristics. These include colonial and cellular morphology, physiological characteristics, antigenic relationship as detected by serological reactions, and cultural and biochemical properties. Depending on these properties, Bergey's Manual of Determinative Bacteriology, 7th ed. (11) included all the known human, animal and saprophytic species of vibrios in the single genus Vibrio. Accordingly, the species were divided into two major groups:

Group I: The aerobic cholera and cholera-like vibrios which are either human pathogens or saprophytes in water and their type species is Vibrio comma.

Group II: The microaerophilic, facultatively anaerobic and the strictly anaerobic species which are either pathogens for man and warm blooded animals or saprophytes in nature and their type species is Vibrio fetus.

The following general description was given to all bacteria included under the genus Vibrio. Short rods, curves, commas, or S-shaped cells, single or united into spirals. Motile by means of a single polar flagellum, rarely two or three flagella in one tuft. Aerobic, facultatively anaerobic and anaerobic species, some ferment sugars and produce acids. Gram negative, indole positive, oxidase positive. They do not sporulate and are devoid of a capsule. This description fits the aerobic human pathogens. However, the facultatively anaerobic or microaerophilic animal pathogens showed some deviation from the general description of the genus. Although morphologically they resemble V. cholera, nevertheless spherical or coccoid forms are encountered in old cultures. They are unable to ferment carbohydrates or produce indole or liquify gelatin and capsulation is still doubtful.

Classification and Nomenclature of Campylobacters

Vibrio fetus (V. fetus) was originally assigned to the genus Vibrio by Smith and Taylor (78). Levy (51), Kuzdas and Morse (47), and Diliello et al. (26) were the first to attempt characterization beyond species identification. They identified different Campylobacters with several characteristics in common. King (45) conducted bacteriological and serological studies of several Campylobacters from human and animal sources. She reported that the human isolates could grow at 42° C and were serologically different from the animal isolates, therefore she called them "related vibrios," but did not clearly assign them taxonomic

position. In another study, King (46) recognized the close similarity between her "related vibrios," Vibrio jejuni of Jones et al. (44), Vibrio coli of Doyle (29) and vibrios of avian origin and assumed that they belong to the same group. The separation of Vibrio fetus into V. fetus var. venerealis and V. fetus var. intestinalis introduced by Florent (37) remained the only recognized division until Bryner and Frank (13) reported the use of catalase reaction to differentiate between V. fetus of Smith and Taylor (78) which was catalase positive and V. bubulus of Florent (36) which was catalase negative. The differentiation based on this characteristic later was found to correlate well with the division of the two vibrios into pathogenic and nonpathogenic groups. Bryner et al. (14), utilizing a biotyping approach, identified for the first time three subdivisions within V. fetus which later came to be known as V. fetus type 1, subtype 1 and type 2. As more and more new parameters were being applied for identification of microorganisms, it became evident that the animal vibrios differed markedly in various respects from the human pathogens.

Basden et al. (4) undertook a study of genetic relationship among six Vibrio strains isolated from bovine, ovine, avian, and human sources. They utilized biochemical tests, deoxyribonucleic acid (DNA) base composition analysis, and the detection of nucleic acid homology by the membrane filter method. They reported varying degrees of genetic homology among vibrios having similar base composition. Also, they found that the bovine isolates have substantially different percentages

of guanine + cytosine ratio and thus they are genetically different from the other strains.

Vibrio fetus continued to be a member of the genus Vibrio till 1963 when Sebald and Veron (67) determined the DNA content of several species of Vibrios and found that the mol % G&C for V. fetus averaged 34.3 (33-35)% while that for classical cholera and halophilic species of Vibrio averaged 47.2 (44-50)%. It was evident that V. fetus had a significantly different DNA base-pair ratio from that of classical species of Vibrio. Furthermore, V. fetus had phenotypic differences from other species of Vibrio which included the inability to ferment or oxidize carbohydrates and a requirement for microaerophilic conditions for growth. Some species of Vibrio can ferment carbohydrates and are facultative anaerobes (73, 74). Hence, Sebald and Veron (67) concluded that V. fetus should be removed from the genus Vibrio and be reclassified as Campylobacter, with C. fetus being the type species of the genus. The new genus included V. fetus var. venerealis and V. fetus var. intestinalis. By combining biochemical properties and serological reactions, Berg et al. (6) reclassified the members of V. fetus and V. jejuni and introduced a different nomenclature. In another taxonomic investigation, utilizing several biochemical and serological tests, Véron and Chatelain (85) rearranged the nomenclature of the Campylobacters and added V. jejuni of Jones et al. (44), V. coli of Doyle (29), V. sputorum of Prevot (62) and V. bubulus of Florent (36) to the new genus Campylobacter.

Smibert (73) in the 8th ed. of Bergey's Manual of Determinative Bacteriology introduced a slightly different concept in classifying the Campylobacters. Three distinct subspecies in the C. fetus group were proposed: C. fetus ssp. fetus, C. fetus ssp. intestinalis, and C. fetus ssp. jejuni. Several different terminologies have been used by others (6, 14, 54) to identify organisms that belong to the C. fetus group. Hence, to avoid misinterpreting what subspecies name may have been used by an investigator, care must be taken to note what nomenclature was used by the particular investigator to classify the organism studied. In a general comprehensive review, Smibert (74) reported and summarized the status of nutrition and classification of the various Campylobacters and emphasized the microaerophilic nature of these organisms. A comparison of some of the different nomenclatures that have been used to classify the C. fetus group is presented in Table 1. The nomenclature proposed by Smibert (73) is most accepted; however, some investigators continue to use the nomenclature proposed by Véron and Chatelain (85).

Briefly, Bergey's Manual of Determinative Bacteriology, Smibert (73) describes C. fetus as follows: C. fetus ssp. fetus (V. fetus var. venerealis, Berg serotype A) causes bovine venereal campylobacteriosis, an endometric infection resulting in abortion, only known to occur in cattle; C. fetus ssp. intestinalis (V. fetus var. intestinalis, Berg serotypes A-2 and B) infects both man and lower animals, producing both disseminated and enteric infections, and sporadic abortion; C. fetus ssp. jejuni (V. fetus var. intestinalis, "related vibrio," V. coli,

Table 1. Comparison of different nomenclatures used to classify the Campylobacter fetus group

Smibert (1974)	Véron and Chatelain (1973)	Florent (1959)	King (1962)	Berg et al. (1971)	Bryner et al. (1962)	Mohanty et al. (1962)
C. fetus subsp. fetus	C. fetus subsp. venerealis	Vibrio fetus var. venerealis	Vibrio fetus	Vibrio fetus serotypes A-1 and A-sub 1	Vibrio fetus type 1 and subtype 1	Vibrio fetus types I and III
C. fetus subsp. intestinalis	C. fetus subsp. fetus	Vibrio fetus var. intestinalis	Vibrio fetus	Vibrio fetus serotypes A-2 and B	Vibrio fetus type 2	Vibrio fetus type II
C. fetus subsp. jejuni	C. jejuni/ C. coli		"Related" vibrios	Vibrio fetus serotype C		

V. jejuni, Montana serotype I or Berg serotype C) produces epizootic abortion in sheep with bacteremia and diarrhea, and enteric infections in children and adults as well as in lower animals. Detailed biochemical tests for identification of campylobacters can be found in the 8th ed. of Bergey's Manual and in the 4th ed. of Anaerobe Laboratory Manual edited by Holdeman et al. (41).

Prior to 1978, very little work had been undertaken towards characterization and/or typing isolates of Campylobacter coli. This is because there are no specimens of the original isolate of Doyle (29) in the ATCC center or elsewhere around the world (74). Also, the recent porcine isolates proved to be nitrate positive whereas Doyle's original isolate of C. coli was reported to be nitrate negative (74). Another reason is the fact that the role of C. coli in swine dysentery has never been clearly established and there are those who believe that C. coli is not the only etiological agent of swine dysentery (31). These two points in particular hampered further investigation in C. coli until its possible role in human enteritis was suggested (30). Similarly, no exhaustive study of the exact characteristics of C. fetus ssp. jejuni has been undertaken before. But the emergence of C. fetus ssp. jejuni as a common cause of acute enteritis in humans (18, 68) and its increased isolation from food animals and birds (39, 74, 80) as well as from companion animals (8, 69) prompted a challenge for the medical as well as the veterinary community to improve existing methods and develop new isolation techniques (23, 68). Characterization and various typing techniques also are being earnestly developed (42).

There are several key characteristics that are used to subspeciate the organisms under the genus C. fetus. Important characteristics used to differentiate C. fetus ssp. jejuni from the other two subspecies include its ability to grow at 43° C but not at 25° C, its sensitivity to nalidixic acid and tolerance to cephalothin and its ability to hydrolyze sodium hippurate. Characteristics used to differentiate C. fetus ssp. fetus from the others include its inability to produce H_2S from Albimi Brucella broth containing cysteine as detected by lead acetate strips and its inability to grow in the presence of 1% glycine. Today the status of biochemical characterization, phage typing and serotyping of C. fetus ssp. jejuni and/or C. coli has been improved and encouraging results are being reported (1, 17, 42, 48, 57, 70).

Habitat and Disease Potential of Campylobacter fetus and Campylobacter coli in Animals and Humans

C. fetus infections are very important in veterinary medicine. In 1913, MacFadyean and Stockman (52) found a microorganism which caused abortion in ewes. In 1918, Smith (75) isolated C. fetus from aborted bovine fetal membranes. C. fetus was first found in man by Vinzent et al. (87) who isolated it from the blood of three pregnant women admitted because of fever of unknown origin, and two of the three women aborted. The agent isolated from the blood cultures was identified as Vibrio fetus (C. fetus ssp. intestinalis) and the authors therefore regarded C. fetus as a new causative agent of abortion (86). C. fetus ssp. fetus has a characteristic reservoir and is responsible for a specific type

of disease. C. fetus ssp. jejuni and C. fetus ssp. intestinalis each has more than one reservoir and can produce more than one type of disease. Several studies recently have been done to determine prevalence of C. fetus ssp. jejuni in different animal populations as part of their normal intestinal flora. Sampling the intestinal contents of 300 apparently normal pigs, investigators recovered C. fetus ssp. jejuni from 182 (61%) of the animals (55). A study of broiler chickens from a live poultry market revealed that 38 of 46 chickens (83%) harbored C. fetus ssp. jejuni in their cecal flora (39). Smith and Muldoon (77) have demonstrated that C. fetus ssp. jejuni can also be isolated from the meat of chickens. Also, various domesticated animals are found to be reservoirs for this organism (74). A recent study revealed that migratory waterfowl commonly shed C. fetus ssp. jejuni in their feces, and of 445 wild ducks sampled, 156 (35%) were positive for C. fetus ssp. jejuni (50).

C. fetus ssp. fetus is primarily associated with disease in cattle in which it is characterized by infertility and occasionally abortion in heifers and cows (20, 79). Its natural habitat is the mucosa of the glans penis, prepuce, and distal portion of the urethra of carrier bulls and the mucosa of the vagina, cervic, uterus and oviducts of the cow (20, 58). Bulls transmit the organism to the vagina of cows during sexual contact. C. fetus ssp. fetus does not survive or grow in the digestive system of a host so that ingesting the organism with food would not be a health hazard (74). This organism has never been identified as an agent responsible for disease in humans (74). Campylobacter

fetus ssp. intestinalis has been cultured from aborted ovine fetuses, but isolations have also been made from ovine blood, bile, and feces (6, 16, 32, 71). There are no reports of isolations from the genitals of male sheep. This subspecies is an inhabitant of the bovine intestinal tract and has been isolated from aborted bovine fetuses and bile (6, 15, 16, 74).

Unlike C. fetus ssp. fetus, C. fetus ssp. intestinalis is a recognized human pathogen. Known reservoirs for this organism include sheep, cattle and humans (74). The organism is known to cause abortion in sheep (24, 38); it sporadically causes abortion in cattle (21) and is the cause of systemic campylobacteriosis in humans (63). As its name implies, C. fetus ssp. intestinalis can survive and multiply within the intestine of a host (74). Its mode of transmission is not venereal but rather by oral ingestion of contaminated material (84). Colonization and infection of C. fetus ssp. intestinalis to digestive organs of cattle and sheep and failure of C. fetus ssp. fetus to do so was documented by Bryner et al. (15). They investigated Vibrio fetus infection of the digestive organs and gallbladders of cattle. Their findings showed that Vibrio fetus type 1 and subtype 1 which correspond to Florent's Vibrio fetus var. venerealis, failed to establish in the digestive tract of cattle, while Vibrio fetus type 2 (Florent's Vibrio fetus var. intesinalis) did.

Campylobacter fetus ssp. jejuni was originally described as the etiologic agent of winter dysentery in cattle (43), although this was

never substantiated. In the veterinary field, C. fetus ssp. jejuni has been clearly shown to be an etiologic agent in enzootic abortion in sheep. Although the organism was originally claimed to be the etiologic agent of winter dysentery in cattle as well as enteritis in dogs and primates (8, 44, 82), the role of this organism in enteric disease in animals is at present not well substantiated. Attempts to produce enteritis in a variety of animals by feeding pure cultures of C. fetus ssp. jejuni have met with limited success. Rates of isolation of C. fetus ssp. jejuni are often similar between diarrheal versus healthy animals of the same species (60). Diseases reported to be produced by C. fetus ssp. jejuni include abortion in sheep (24, 74); hepatitis in poultry (40, 56); acute gastroenteritis in humans (18, 23, 68), monkeys (82, 83), cats and dogs (8, 9); and dysentery in cattle (43, 44). Like C. fetus ssp. intestinalis, C. fetus ssp. jejuni can survive and grow in the intestinal tract of its host and transmission of the organism is by the oral route (74). C. fetus ssp. jejuni is much more commonly associated with human disease than is C. fetus ssp. intestinalis. This may be partially attributed to the fact that C. fetus ssp. jejuni occurs more widely in nature than the other subspecies. Besides being present in infected animals, C. fetus ssp. jejuni is often isolated from the feces of normal young swine (55), cattle (15, 74), sheep (71), goats (73, 74), poultry (39, 74) and several species of wild birds (50, 72). As these animals mature, smaller numbers of C. fetus ssp. jejuni are generally present as part of their intestinal flora (74). Pets, including

dogs (8, 9) and cats (9, 80), have been suggested as sources of C. fetus ssp. jejuni for human infection.

Campylobacter fetus ssp. jejuni's ability to produce abortion has been well-documented (5, 52, 74). Firehammer and Hawkins (33) studied the pathogenicity of different ovine bile isolates in pregnant ewes using the oral and the intravenous routes of inoculation and showed that almost 80% of the ewes tested aborted, thus providing substantial evidence of the ability of vibrios of bile origin to cause abortion in sheep. Firehammer et al. (34) demonstrated bacteremia in pregnant ewes and young lambs after experimental oral infection with Vibrio fetus. Firehammer and Myers (35) reported producing pathologic changes in the intestinal tracts of challenged calves but little actual diarrhea; they have made numerous isolations of C. fetus ssp. jejuni from diarrheic calves.

Levy (51) was the first to associate animal vibrios with human gastroenteritis in Illinois. Levy described a large epidemic of febrile gastroenteritis in adults. An organism he described as vibrio-like, later called Levy's vibrios by King (45), could be seen in smears of half of the cases. The same organisms were isolated from the blood of many of the patients. During 1957, King (45) reported seven additional cases of human enteric infection due to Vibrio fetus and four cases due to vibrio species closely related to Vibrio fetus. She designated the latter strains as "related vibrios." During 1972, Bokkenheuser (10) reported 10 additional cases of Vibrio fetus infection in man. Most of these later were identified as C. fetus ssp. intestinalis. In 1971,

Cooper and Slee (22) reported a case of acute septicemia with intestinal infection in an infant girl from whose blood and stool Vibrio fetus var. intestinalis had been isolated.

Avian vibrionic hepatitis, an infectious disease of chickens characterized by degenerative changes in the liver and heart, has been known for a long time (40). The causative agent was found by McGehee et al. (53) to be a vibrio organism. Isolation of the organism from the bile of sick birds was reported by Peckham (56). Similar organisms were obtained by Winterfield et al. (91) from feces of laying hens on different occasions.

Swine dysentery is a serious disease with the death rate ranging from 40 to 60% in young pigs and 10 to 20% in feeder pigs (89). Doyle (27, 29) described C. coli as a cause of dysentery in pigs. Birrell (7) noted that vibrios were in constant association with swine dysentery but questioned their role in production of the disease. Peckham (56) and Hofstad (40) considered it as a cause of hepatitis in poultry. Some regarded it as an intestinal commensal both in pigs and poultry (25, 81). Experimental infection of pigs with vibrio cultures isolated from spontaneous cases by Doyle (28) was successful when the organisms were given in gastric mucin. When the hogs were given the cultures without mucin, they remained uninfected. Unsuccessful trials of oral infection of gnotobiotic pigs with Vibrio coli strains isolated from healthy as well as sick pigs were reported by Andress et al. (3). Nevertheless, the organisms were recovered from the feces of the clinically normal

experimental gnotobiotic pigs. Similar results of an experiment conducted with conventional pigs were reported by Andress and Barnum (2). This might suggest the existence of nonpathogenic strains of Vibrio coli in normal pigs, the establishment of some sort of commensalism or symbiosis between Vibrio coli and its swine host, or additional factors such as viruses or Treponema (31) are necessary for the production of dysentery.

Experimental Infection of Laboratory Animals with Campylobacters

The importance of laboratory animals as experimental models for the purpose of studying the pathogenesis, pathology, diagnosis, and treatment of different illnesses as well as the determination of the pathogenicity and the relative virulence of many pathogenic microorganisms has been recognized since the beginnings of medical sciences. Campylobacters isolated from domestic animals display different degrees of pathogenicity for laboratory animals. Smith (75) found that Vibrio fetus was nonpathogenic for nonpregnant guinea pigs, normal rabbits, rats, and mice by the intraperitoneal route. Lerche (49) indicated that pregnant guinea pigs can be successfully infected with Vibrio fetus by the intraperitoneal, conjunctival, and the subcutaneous routes resulting in abortion. Ristic and Morse (64) and Ristic et al. (65) investigated the infectivity of Vibrio fetus in pregnant and nonpregnant guinea pigs by the intraperitoneal, intravaginal, oral, and subcutaneous routes. The authors indicated that a high percentage of the pregnant guinea pigs

contracted infection regardless of the route of inoculation. The resultant abortion rate was 47-78% and Vibrio fetus was recovered from the uteri, fetuses and gall bladders of the infected guinea pigs. Ristic et al. (65) reported that male hamsters could be infected with Vibrio fetus by the intraperitoneal route and the organism was recovered in pure culture from the testes. These infected males were able to transmit the infection to female hamsters during coitus.

Bryner et al. (12) investigated the pathogencity of three strains of Vibrio fetus, (type 1, subtype 1 and type 2) in rabbits, guinea pigs, and white mice using the intraperitoneal, subcutaneous and intravenous routes of inoculation. According to their findings, type 1 was not recovered from any of the mice or rabbits and from only one of 34 guinea pigs. Subtype 1 was recovered from one of 40 mice tested. Type 2 was recovered from the feces of five of eight rabbits, from two of six guinea pigs, and from 23 of 28 mice tested. Type 2 killed 50% of the nonpregnant guinea pigs and 13% of the mice. It is apparent that mice are the most susceptible and nonpregnant guinea pigs are the least susceptible to infection with type 2. Type 1 and subtype 1 failed to establish infection in the digestive organs of the tested animals. Type 2 strains had the ability to infect the gall bladder of animals injected parenterally and most probably were carried into the intestines through the bile duct and later excreted with the feces (12).

Plastridge and Williams (59) successfully cultivated Vibrio fetus in the allantoic fluid of 12- to 14-day old embryonating chicken eggs

with fatal result. It was also found by Webster and Thorp (88) that Vibrio fetus infection produces congestion and hemorrhage of chorio-allantoic membrane and occasionally necrosis of the liver, spleen, kidney, gizzard, and brain of adult chickens. Adult chickens do not generally show signs of illness after oral challenge, yet the organisms may be invasive (19). However, in 3-day old chickens, oral infection with C. fetus ssp. jejuni resulting in diarrhea in as high as 80% of the chickens was reported (66). Prescott and Karmali (61) observed no more than minimal signs in puppies challenged with 2.5×10^{10} organisms.

The pathogenicity of Vibrio jejuni for laboratory animals was studied by Jones et al. (44). When the intraperitoneal route of inoculation was used, nonpregnant guinea pigs and white rats proved resistant to Vibrio jejuni infection. Some difference in the degree of pathogenicity to rabbits and white mice between various strains of Vibrio jejuni was found. Some strains produced initial, well-marked, febrile reactions when injected intravenously into rabbits, followed later by catarrhal inflammation of the small intestine and diarrhea. The vibrios were recovered from the peripheral blood and the intestinal mucosa. Other strains failed to produce infection of rats and normal rabbits by the oral route. Doyle (29) studied Vibrio coli infection in rabbits, guinea pigs, white rats, mice, and chickens using the parenteral routes of inoculation. His results indicated that Vibrio coli is nonpathogenic to these animals.

Pathogenetic Mechanisms of *Campylobacters*

Very little is known about the pathogenesis mechanisms of these organisms. In fact, very few studies dealing with this subject have been reported and the demand for more investigation is urgent. However, Butzler and Skirrow (19) investigated the ability of thermo-labile and thermo-stable enterotoxin production in monolayer adrenal tumour cell cultures and in 4-day-old white Swiss mice respectively by "related campylobacters" (*C. fetus* ssp. *jejuni*), and concluded that enterotoxin production is a feature of only a few strains. Furthermore, they studied the invasive ability of these organisms to chicken embryo cells and indicated that "related campylobacters" are pathogenic mainly by virtue of their direct invasive ability. At this time, one of the greatest needs in the field of *Campylobacter* research is the characterization of an animal model in which *Campylobacter* enteritis can be produced to serve as a test for pathogenicity of *C. fetus* ssp. *jejuni* strains isolated from the numerous affected hosts.

CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF *CAMPYLOBACTER FETUS* SUBSPECIES *JEJUNI*/ *CAMPYLOBACTER COLI* FROM MAN AND ANIMALS

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Abstract

One hundred one Campylobacter isolates from human and various animal sources were screened for the basic characteristics of Campylobacter fetus ssp. jejuni and Campylobacter coli and 51 strains were selected for further characterization. With the aid of 21 cultural and biochemical tests, the 51 strains were compared with 6 reference strains representing C. fetus ssp. jejuni, C. fetus ssp. fetus and C. fetus ssp. intestinalis. Colony type, cellular morphology, growth at 25° and 43° C, sensitivity to nalidixic acid and cephalothin, tolerance to 4% triphenyl tetrazolium chloride (TTC) and to 0.001% brilliant green (BG) were the tests used to distinguish strains of the jejuni/coli group from ssp. fetus and ssp. intestinalis. Growth at 30.5° and 45.5° C and hydrolysis of sodium hippurate separated strains of jejuni/ coli into 2 groups representing C. fetus ssp. jejuni and C. coli respectively. Seven atypical strains with intermediate characteristic sharing some characteristics with each of group 1 and 2 were also recognized. Of the 51 selected strains, 37 were identified as C. fetus ssp. jejuni, 9 as C. coli and 7 had intermediate characteristics. Tolerance to TTC, and to 1% glycine and DNase production further divided the strains of group 1 into 7 subgroups and those of group 2 into 4 subgroups and the atypical strains into 4 subgroups. This study indicated that C. fetus ssp. jejuni and C. coli are very closely related and the tests presently available are not sufficient to separate them into 2 distinct species.

Introduction

Campylobacter fetus subspecies jejuni (C. fetus ssp. jejuni) was formerly known as Vibrio jejuni (25), and Campylobacter coli was known as Vibrio coli (15). Numerous reports describing organisms similar to C. fetus ssp. jejuni, C. coli and other Campylobacters from sick and healthy animals have appeared in the literature (1, 3, 8, 15, 18, 25, 32, 38, 43). Also, Campylobacter organisms having characteristics similar to those of C. fetus ssp. jejuni were reported from avian species (22, 35, 39, 42). Recently Dekeyser et al.'s (13) filtration technique and Skirrow's (36) selective medium have greatly facilitated the recovery of C. fetus ssp. jejuni and C. coli from human stools (4, 5, 9, 17, 26, 29).

In retrospect, Levy (31) and King (27) were the first to point out the close similarity between C. fetus ssp. jejuni, C. coli, avian Campylobacters and Campylobacter strains isolated from cases of human enteritis to which they assigned the names Vibrio-like and "related Vibrios" respectively. However, the relationship among most of the above isolates was unclear and consequently they appeared under various names. Some of those names are: "Vibrio jejuni" (25), "Vibrio coli" (15), "Vibrio of avian hepatitis" (35), "Vibrio-like" (31), "related vibrios" (27), and "Vibrio fetus var. intestinalis" (38).

Early investigators reported 3 biotypes for C. fetus, but they did not attempt to identify C. fetus ssp. jejuni (7, 14, 28, 34). Later studies (41) identified it as a subspecies of C. fetus while Véron and

Chatelain (44) proposed a species status for this organism. The present literature (37, 45) indicates that C. fetus ssp. jejuni isolates from human, avian and various animal species may not constitute a biochemically homogenous group. Also, C. fetus ssp. jejuni and C. coli strains have numerous biochemical characteristics in common making differentiation and typing of new isolates difficult.

The purpose of this study was to investigate the cultural and biochemical characteristics of 51 Campylobacter strains isolated from cattle, sheep, dogs, pigs, chickens, monkeys, and humans identified as C. fetus ssp. jejuni and/or C. coli and to attempt to organize them into distinct groups or biotypes.

Materials and Methods

Test strains. One hundred one isolates of Campylobacter of human and animal origin were screened by eight differential criteria; i.e., morphology, catalase production, H_2S production by the sensitive (lead acetate paper strip) or insensitive (SIM medium) test, growth at 25^{o} and 43^{o} C, sensitivity to nalidixic acid and resistance to cephalothin. Fifty-one strains which had the characteristics of C. fetus ssp. jejuni or C. coli as per the 8th edition of Bergey's Manual of Determinative Bacteriology (41) and the classification of Holdeman et al. (23) were selected for further study as test strains. As the above differential tests are only adequate to differentiate C. fetus ssp. jejuni from subspecies fetus and subspecies intestinalis but not from C. coli, the test strains were referred to as the "jejuni/coli group."

Reference strains. Six reference strains, designated 482, 1289, 436, 1256 of bovine origin, 958 and 652 of ovine origin were included in the study for comparison. Strains 958 and 652 were identified as C. fetus ssp. jejuni and were referred to as the "jejuni reference group." Strains 482 and 1289 were identified as C. fetus ssp. fetus (subspecies venerealis) of Véron and Chatelain (44) and 436 and 1254 were identified as C. fetus ssp. intestinalis (subspecies fetus) of Véron and Chatelain (44). These were referred to as the "nonjejuni reference group."

All the strains were collected by the second author at the National Animal Disease Center (NADC) at Ames, Iowa. Their strain numbers, origins and sources are presented in Table 1.

Culture media. Blood agar (BA) was prepared from brain heart infusion agar (BBL, Cockeysville, Maryland, Catalog No. 11065) with 10% defibrinated bovine blood added aseptically at the time of pouring into petri plates.

Brucella agar and Brucella agar overlay. Brucella agar (BrA) was prepared from Brucella Agar Dri-Form (Gibco Diagnostics, Madison, Wisconsin, Catalog No. M08400) according to directions and poured into petri plates. Brucella agar overlay (BrAO) was prepared as BrA, dispensed into 15x100 mm tubes (3 ml/tube), and used in place of overlay agar to prepare bacterial growth lawns for antibiotic sensitivity tests.

Brucella broth. Brucella broth (BB) was prepared from Brucella Broth (Albimi) Dri-Form (Gibco Diagnostics, Catalog No. M08400) as

Table 1. Reference and test strains used in this study

Serial No.	Lab. No.	Origin & Habitat	Isolation Date	Source	Identification
Reference Strains					
1	482	Bull semen	1960	Maryland	ssp. fetus
2	1289	Bovine uterus	1976	Iowa	ssp. fetus
3	436	Bovine fetus	1956	Maryland	ssp. intestinalis
4	1254	Bovine fetus	1962	New York	ssp. intestinalis
5	958	Ovine fetus	1964	Utah	ssp. jejuni
6	652	Ovine fetus	1962	Colorado	ssp. jejuni
Test Strains					
1	746	Bovine gallbladder	1963	Iowa	ssp. jejuni
2	747	Bovine gallbladder	1963	Iowa	ssp. jejuni
3	748	Bovine gallbladder	1963	Iowa	C. coli (atypical)
4	750	Bovine gallbladder	1963	Iowa	ssp. jejuni
5	751	Bovine gallbladder	1963	Iowa	ssp. jejuni
6	753	Bovine gallbladder	1963	Iowa	ssp. jejuni (atypical)
7	754	Bovine gallbladder	1963	Iowa	ssp. jejuni
8	755	Bovine gallbladder	1963	Iowa	ssp. jejuni
9	756	Bovine gallbladder	1963	Iowa	ssp. jejuni
10	764	Bovine gallbladder	1963	Iowa	ssp. jejuni
11	767	Bovine gallbladder	1963	Iowa	ssp. jejuni
12	776	Bovine gallbladder	1963	Iowa	ssp. jejuni
13	778	Bovine gallbladder	1963	Iowa	ssp. jejuni
14	890	Calf feces	1970	Colorado	ssp. jejuni
15	657	Ovine uterus	1962	Montana	ssp. jejuni (atypical)
16	917	Ovine fetal	1961	Utah	ssp. jejuni
17	1114	Ovine fetus	1967	Montana	ssp. jejuni
18	1220	Ovine fetus	1959	Colorado	ssp. jejuni

19	1269	Ovine uterus	1973	Idaho	ssp. jejuni (atypical)
20	426	Swine kidney	1956	Maryland	C. coli
21	440	Swine heart blood	1957	Maryland	C. coli
22	1143	Swine feces	1971	Iowa	C. coli
23	1380	Swine fetal stomach	1978	Iowa	C. coli
24	1207	Human blood and uterus	1971	California	C. coli
25	1211	Human stool	1971	California	ssp. jejuni (atypical)
26	1299	Human baby stool	1977	So. Africa	ssp. jejuni
27	1334	Human blood	1977	So. Africa	ssp. jejuni
28	1349	Human stool	1977	New York	ssp. jejuni (atypical)
29	1369	Human stool	1977	New York	ssp. jejuni
30	1370	Human stool	1977	New York	ssp. jejuni
31	1145	Bird intestine	1971	Iowa	ssp. jejuni
32	1268	Chicken feces	1958	New York	ssp. jejuni
33	1350	Chicken feces	1977	So. Africa	ssp. jejuni
34	1351	Chicken feces	1977	So. Africa	ssp. jejuni
35	1352	Chicken feces	1977	So. Africa	ssp. jejuni
36	1353	Chicken feces	1977	So. Africa	C. coli
37	1355	Chicken feces	1977	So. Africa	C. coli (atypical)
38	1373	Chicken feces	1978	New York	ssp. jejuni
39	1374	Chicken feces	1978	New York	ssp. jejuni
40	1375	Dog feces	1978	Colorado	ssp. jejuni
41	1379	Dog feces	1978	Colorado	ssp. jejuni
42	1383	Dog feces	1978	Colorado	ssp. jejuni
43	1384	Dog feces	1978	Colorado	ssp. jejuni
44	1385	Dog feces	1978	Colorado	ssp. jejuni
45	1386	Dog feces	1978	Colorado	ssp. jejuni
46	1387	Dog feces	1978	Colorado	ssp. jejuni
47	1388	Dog feces	1978	Colorado	C. coli
48	1440	Monkey feces	1980	Illinois	C. coli
49	1441	Monkey feces	1980	Illinois	ssp. jejuni
50	1442	Monkey feces	1980	Illinois	C. coli
51	1443	Monkey feces	1980	Illinois	ssp. jejuni

directed and dispensed into 250 ml flasks (100 ml/flask) or 16x150 mm tubes (10 ml/tube).

Brilliant green agar. Brilliant green agar (BG) was prepared with 0.01g powdered brilliant green (Allied Chemical Corporation, New York, N.Y.), added to 44g Brucella Agar Dri-Form, dissolved in 1000 ml H_2O, autoclaved at 121° C for 15 min and poured into petri plates.

DNase media. Plain DNase medium was made from 42 g of DNase test agar (Difco Lab., Detroit, Michigan, Catalog No. 0632), dissolved in 1000 ml distilled water by heating to a boil, autoclaved at 121° C for 15 min and poured into petri plates. To a similar amount of the DNase agar toluidine blue (Fischer Scientific Co., Fair Lawn, New Jersey) was added to a final dye concentration of 0.01% resulting in a clear toluidine blue DNase medium, and was poured into petri plates.

Basal medium. Semisolid Brucella agar medium consisted of 29g Brucella Broth (Albimi) Dri-Form and 1.6g agar was dissolved in 1000 ml H_2O, dispensed into 16x150 mm screw cap tubes (10ml/tube) and sterilized by autoclaving.

Basal medium with supplements. Five hundred ml amounts of basal medium were prepared in flasks. Aqueous solutions of D-glucose, phenol red, NaCl, glycine (Eastman Organic Chemicals, Rochester, N.Y.), KNO_3 (Mallinckrodt Chemical Works, St. Louis, Missouri), cysteine (Calbiochem-Behring Corp., La Jolla, California), and Bacto-oxgall (Difco Lab) were prepared separately as supplements. A specific amount of each supplement was added to 500 ml of basal medium to produce final concentrations of 8% glucose, 3.5% NaCl, 1% glycine, 1% KNO_3, 1% Oxgall, 0.02% cysteine,

or 1% glucose with 0.002% phenol red as an indicator in the supplemented media as described by Holdeman et al. (23). Each medium was dispensed into 16x150 mm tubes (10ml/tube) and sterilized by autoclaving for 15 min at 121° C.

Sulfide-Indole-Motility agar medium (SIM). Using BBL products and the method described by MacFaddin (33), SIM medium was prepared and dispensed into 16x150 mm tubes and solidified in the vertical position.

Triphenyl Tetrazolium Chloride (TTC) strips. A 4% solution of TTC (Nutritional Biochemicals Corporation, Cleveland, Ohio) in distilled H_2O was prepared and used to saturate 0.7x7 cm strips of Whatman no. 1 filter paper to obtain TTC impregnated strips as described by Skirrow and Benjamin (37).

All the media in plates were stored at 4° C until used but no longer than one week. All the media in tubes were stored at room temperature (23-25° C) and used within 2-3 weeks.

Conditions of cultivation. Agar plates were incubated in desiccators in microaerophelic conditions (5% O_2, 10% CO_2, 85% N) as recommended by Fletcher and Plastridge (21). Liquid and semisolid media were incubated in atmospheric air. Routine incubation temperature for plates and tubes was 37° C except when 25°, 30.5°, 43°, and 45.5° C were used for growth temperature tests. Inoculated plates were incubated from 2-5 days; and tubes from 3-7 days except the 1% glycine, the 8% glucose, and the 3.5% NaCl tubes which were discarded as negative only after 10 days. All tests were run in duplicate and any test giving dissimilar results in a pair was rerun twice to determine reproducibility of the given

results. Routinely, 2 plates of plain BrA or 2 tubes of plain basal medium were inoculated and included as positive controls for growth. Growth was assessed visually by comparing test units with the controls and rated as (+) for good to excellent growth, (±) for limited growth, (-) for no growth, and (v) for variable results. The inoculum was always a broth suspension of 24-48 h growth on BrA adjusted to an optical density (OD) of 0.2 at 550 nm using a spectrophotometer (Spectronic 20 model 340 Bausch and Lomb). 0.25 ml ($\simeq 2.5 \times 10^7$ viable cells) of the above suspension was the inoculum size for each tubed medium while 2 drops from 1 ml serological pipette (0.05 ml $\simeq 5 \times 10^6$ viable cells) were streaked on the surface of each plate.

Microscopic examinations. With the aid of a dissection microscope at 20x magnification, colonies from 48 h cultures were examined for appearance on the surface of BA and BrA plates.

Cellular morphology and motility. Cell morphology and motility of 48, 72, and 96 h cultures suspended in broth were examined under a phase contrast microscope at 1212x magnification. The cellular morphology of representative strains was further studied under the EM.

Gram and flagellar staining. Gram staining was performed on smears from 48 h cultures on BrA by the Hucker's modification method (11). Possession of flagella and their location on the cells were investigated using smears stained with Leifson's flagella stain as described by CDC (11). Light microscope at 900x magnification was used to view the staining characteristics and flagellation.

Growth and biochemical tests. Ability to grow in basal media containing 1% glycine, 3.5% NaCl, 1% oxgall or 8% glucose, and ability to ferment 1% glucose in basal medium with 0.002% phenol red as an indicator were investigated according to Holdeman et al.'s technique (23). Catalase production was evaluated by the quantitative method of Bryner and Frank (6) using 3-day-old cultures in basal medium and 3% hydrogen peroxide. Oxidase activity was determined by Kovac's modification method (11) with 0.5% solution of N-tetramethyl-P-phenylene diamine dihydrochloride (Eastman Kodak Co., New York, N.Y.). Nitrate and nitrite reduction was tested by the CDC method (11) using 3-day-old cultures in basal medium supplemented with 1% KNO_3. H_2S production in cysteine-containing medium was detected by blackening of lead acetate paper (Fischer Scientific Co.), (sensitive test) and H_2S production in iron containing SIM medium was detected by blackening of the butt (insensitive test) according to Holdeman et al. (23). Tolerance to 0.001% brilliant green was determined by testing for growth on BG plates. The test for tolerance to 4% TTC was performed in a similar manner as described by Skirrow and Benjamin (37) using TTC impregnated strips and BrA medium in place of BA. Growth inhibition zones from strip to edge of growth were measured in mm and any strain showing inhibition zone less than 2 mm was considered tolerant. Ability to hydrolize sodium hippurate was tested by the rapid hippurate hydrolysis method of Hwang and Ederer (24) using 1% aquous solution of sodium hippurate (Nutritional Biochemical Corporation) and a solution of 3.5g ninhydrin (Sigma Chemical Co., St. Louis, Missouri) in 100 ml of a

1:1 mixture of acetone and butanol. Results of the hippurate test were read at the end of a 3 min period instead of 10 min. Any color shade less than a deep purple was considered negative. Production of extracellular DNase (enzyme) that hydrolizes deoxyribonucleic acid incorporated in an agar medium was tested by both the standard test and the Schreier's Toluidine blue modification technique as described by Black et al. (2). Appearance of a clear zone around the colony indicated a positive reaction.

Susceptibility to Nalidixic Acid and Cephalothin. One ml broth suspension of 48 h Campylobacter culture standardized to 0.2 OD was mixed with 3 ml of BrAO at 48° C and poured onto BrA plate. After hardening, a 30 mcg disc of nalidixic acid or cephalothin (BBL sensidisc) was placed in the center of the plate and incubated microaerophilically at 37° C for 48 h. Zone of inhibition from edge of disc to confluent growth was measured in mm. Strains with inhibition zone $\geq$ 10 mm were considered sensitive.

Temperature growth range. Ability to grow at 25°, 30.5°, 37°, 43°, and 45.5° C was investigated by streaking duplicate BrA plates and incubating them microaerophilically at the specified temperatures up to 5 days.

Results

Colony types and cellular morphology. Two colony types were found: one referred to as the "discrete type," was a smooth, round,

convex, entire with an opaque center, glistening edge and whitish-grey color (Fig. 1-A); the second type was smooth, amoeboid, flat, swarming with bluish-grey color (Fig. 1-B). One C. fetus ssp. jejuni reference strain and 31 of the test strains displayed the first colony type. The nonjejuni reference strains produced only the first colony type. One C. fetus ssp. jejuni reference strain and 20 test strains produced the second colony type (Table 2).

The predominant cell types from 48 h cultures were the incomplete S-shapes, observed in 54/57 strains and the comma-shapes, observed in 40/57 strains (Fig. 2-A & B). Spirals were occasionally observed in 21/57 strains after 30 h incubation (Fig. 2-C). In older cultures (72-96 h) coccoidal cells predominated in the test strains (Fig. 2-D). For the nonjejuni reference strains coccoidal cells were rarely seen, even in older cultures. Of the 57 strains studied, 52 (91%) were actively motile (Table 2). Five strains (9%) were either completely nonmotile or had very few motile cells. Trials of selection for purely motile cells from the strains exhibiting poor motility were unsuccessful. Flagella were observed in 56 of the strains (98%). A few flagellated cells were observed in 4 of the 5 nonmotile strains. All the Campylobacter strains studied were consistently gram negative, but they were better viewed under the phase contrast microscope.

Growth and biochemical responses. In 10 of the 21 tests conducted, the responses of all the strains were identical; i.e., either 100% positive or 100% negative. Results of the remaining 11 tests are given (Table 3). Based on the results of the biochemical tests, the

Fig. 1. Colony types of *Campylobacter fetus* ssp. *jejuni*/*C. coli*

A. Discrete convex colonies found in 32/53 strains

B. Swarming (ameboid) growth found in 21/53 strains

Both discrete and swarming colonies were encountered in a number of strains

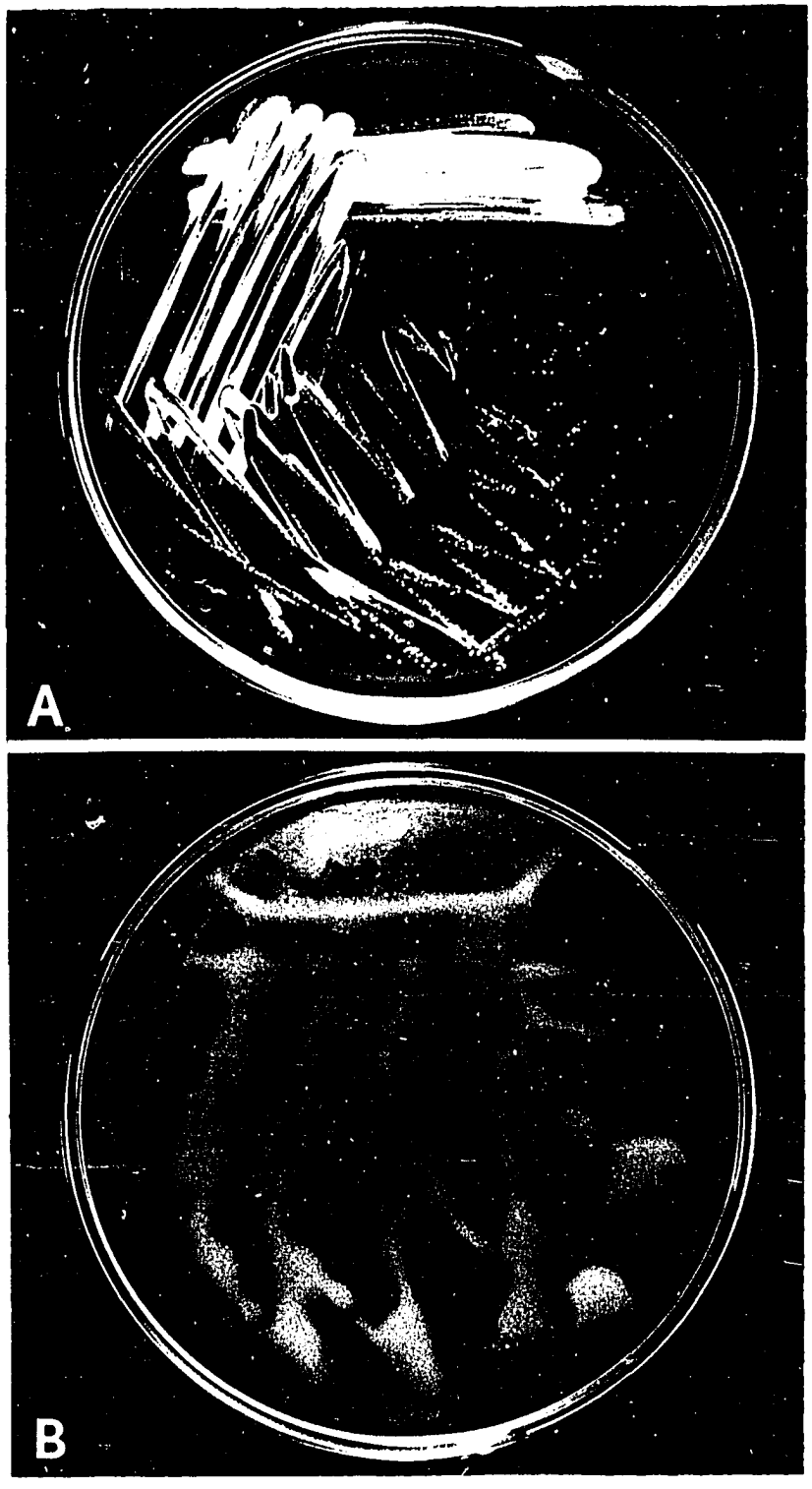

A
B

Table 2. Motility, flagellation and colony type of Campylobacter strains studied

Strains	No. Tested	Motility		Flagellation		Colony Type	
		M[a]	Non-M[b]	F[c]	Non-F[d]	Discrete	Swarming
Reference Strains							
C.f. ssp. fetus	2	2	0	2	0	2	0
C.f. ssp. intestinalis	2	2	0	2	0	2	0
C.f. ssp. jejuni	2	2	0	2	0	1	1
Test Strains							
C.f. ssp. jejuni/C. coli	51	46	5	50	1	31	20
Total	57	52	5	56	1	36	21

[a]M, Motile.

[b]Non-M, Non-motile.

[c]F, Flagellated.

[d]Non-F, Non-flagellated.

Fig. 2. Cell types found in one or more of the Campylobacter fetus ssp. jejuni/C. coli strains

A. Comma-shaped cell with single polar flagellum observed in 40/57 strains

B. S-shaped cells with single polar flagellum observed in 54/57 strains

C. Spiral cell with bipolar flagella observed in 21/57 strains

D. Coccoid cells with and without flagella observed in all strains after long incubation periods (96+ hours)

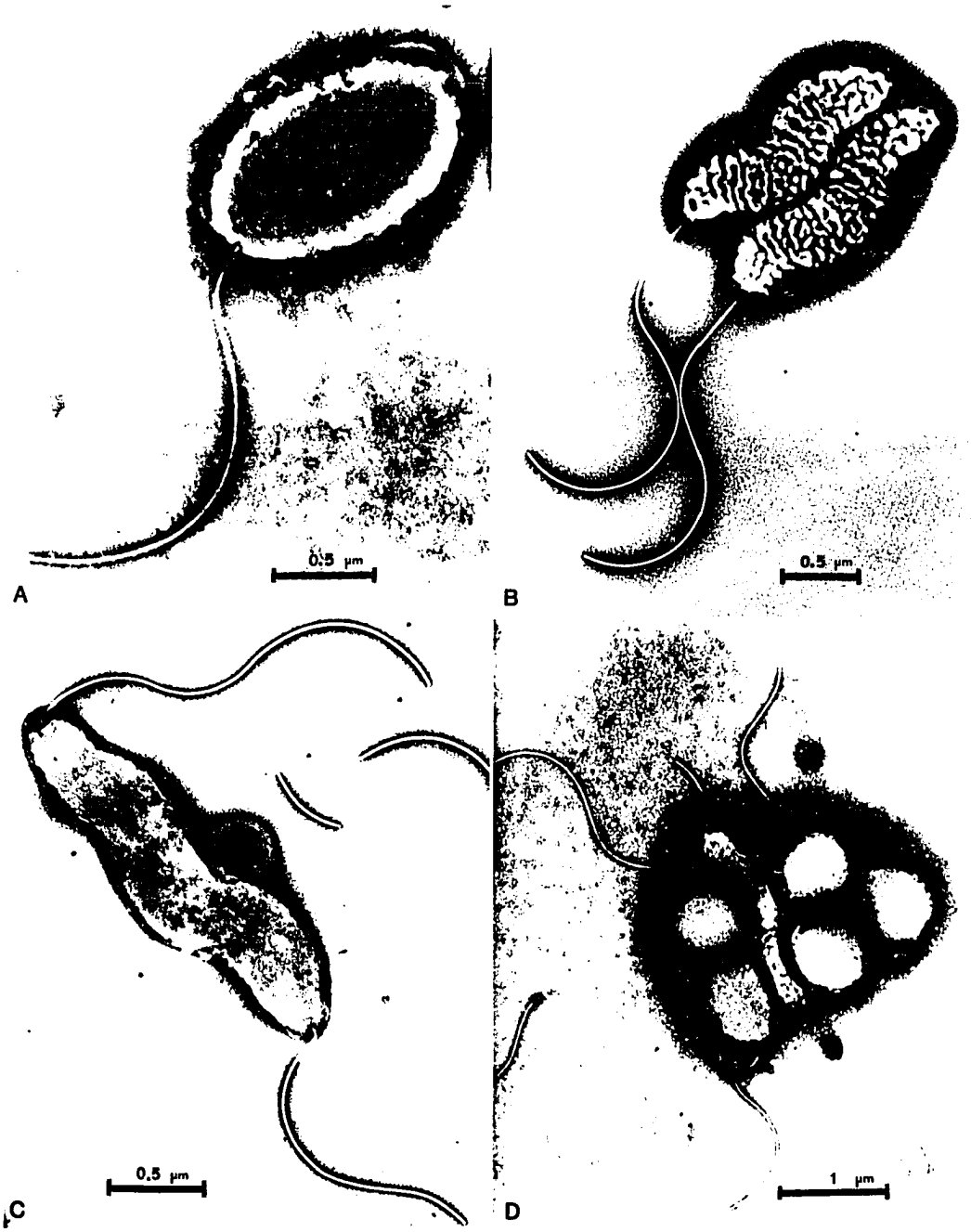

0.5 µm
A
0.5 µm
B
0.5 µm
C
1 µm
D

Table 3. Response to 21 bacteriological and biochemical tests by Campylobacter strains studied

	Test	No. positive strains/no. strains tested			
		Reference Strains			Test Strains
		fetus	intestinalis	jejuni	jejuni/coli
1	Catatalase activity	2/2	2/2	2/2	51/51
2	Oxidase activity	2/2	2/2	2/2	51/51
3	Nitrate reduction	2/2	2/2	2/2	51/51
4	Nitrite reduction	0/2	0/2	0/2	0/51
5	Tolerance to 1% oxgall	2/2	2/2	2/2	51/51
6	Tolerance to 3.5% NaCl	0/2	0/2	0/2	0/51
7	H_2S (sensitive test)	(1)[a]/2	(2)/2	2/2	51/51
8	H_2S (nonsensitive test)	0/2	0/2	0/2	0/51
9	1% Glucose fermentation	0/2	0/2	0/2	0/51
10	Tolerance to 8% glucose	0/2	0/2	0/2	0/51
11	Tolerance to 1% glycine	0/2	2/2	0/2	39/51
12	Hippurate hydrolysis	0/2	0/2	2/2	40/51
13	Tolerance to 0.001% Brilliant green	2/2	2/2	0/2	2+(4)/51
14	Tolerance to 4% TTC[b]	0/2	0/2	2/2	35/51
15	DNase production	0/2	0/2	1/2	14/51
16	Growth at 25° C	2/2	2/2	0/2	0/51
17	Growth at 30.5° C	2/2	2/2	0/2	10/51
18	Growth at 43° C	0/2	(1)/2	2/2	51/51
19	Growth at 45.5° C	0/2	0/2	0/2	2+(3)/51
20	Nalidixic acid (30mcg) S[c]	0/2	0/2	2/2	51/51
21	Cephalothin (30mcg) S[c]	2/2	2/2	0/2	1/51

[a] ( ) = Parenthetic numbers represent strains showing weak or variable response to the particular test.

[b] TTC, Triphenyl tetrazolium chloride.

[c] S, Sensitivity.

test strains can be divided into 2 groups (1 and 2) representing C. fetus ssp. jejuni and C. coli. Between these 2 groups appeared a few strains with intermediate characteristics. All the bovine strains fell into the C. fetus ssp. jejuni group while all the swine strains fell into the C. coli group. Strains from other hosts were distributed randomly between the 2 groups.

Thirty-seven strains were found to belong to group 1, 9 to group 2 and 7 atypical strains exhibited intermediate characteristics. Seven subgroups were recognized in group 1, 4 subgroups were recognized in group 2 and 4 subgroups were recognized within the strains with intermediate characteristics (Table 4). Subgroup 5 (TTC tolerant, glycine positive and DNase negative) and subgroup 6 (TTC sensitive, glycine positive and DNase negative) were the most prevalent subgroups. No correlation between subgroups and hosts of origin was observed.

Discussion

Campylobacter fetus ssp. jejuni/C. coli colonies had a tendency to spread and swarm on surface of agar plates as previously reported (32, 38) in contrast to the nonspreading discrete colonies of other campylobacters. Also, the ability of the C. fetus ssp. jejuni/C. coli cells to undergo rapid transformation into coccoidal forms in older cultures (32, 41) provides an additional means for distinguishing these organisms from other campylobacters.

On the basis of the biochemical results, the 3 subspecies of Campylobacter fetus and C. coli share 10 characteristics (tests 1-10,

Table 3) which are of no help in differentiating any of them from the others. However, 5 other tests proved to be useful as differential tests. These are: growth at 25° and 43° C, sensitivity to naladixic acid and to a lesser degree tolerance to TTC and brilliant green. In addition, sensitivity to cephalothin gave a clear cut differentiation between the jejuni/coli group and the other 2 subspecies of C. fetus. Eugonic growth at 43° C was a consistent feature of the jejuni/coli group but they completely failed to grow at 25° C which agrees with previous reports (23, 27, 28, 38). On the contrary the nonjejuni reference strains grow at 25° C but not at 43° C. We observed that a few strains of C. fetus ssp. intestinalis had a tendency to grow sparsely at 43° C. For differential purposes, growth at the lower temperature limit of 25° C is a reliable test. Members of the nonjejuni reference group are brilliant green tolerant and TTC sensitive whereas the majority of the jejuni/coli group are brilliant green sensitive (89%) and TTC tolerant (70%). Campylobacter coli was reported to be BG tolerant and C. fetus ssp. jejuni BG sensitive (16, 44), which might allow their differentiation. But this was disputed by others (12, 37) who reported complete intolerance of both organisms to 0.001% BG which agrees with our findings. The result that all the strains of the non-jejuni reference group are TTC sensitive is in conformity with results of previous investigators (37). But the finding that 16 strains of the jejuni/coli group (30%) also are TTC sensitive makes the test less reliable for the purpose of differentiating the subspecies of C. fetus.

Contrary to one report (44) and in agreement with several other reports (18, 23, 41) our results indicate that Campylobacter fetus and C. coli do not produce H_2S in iron containing medium (insensitive test). Contrary to early reports that suggested that C. fetus ssp. jejuni is exclusively glycine tolerant, our results showed that 25% of the test strains studied were intolerant to 1% glycine. Similar findings were reported earlier for both C. fetus ssp. jejuni and C. coli (12, 18, 19, 21, 30, 32). We think that this high percentage of glycine intolerance can only be a reflection of an intrinsic characteristic, so the occurrence of glycine negative strains of C. fetus ssp. jejuni and C. coli must always be taken into account.

Growth in 8% glucose as a differential test between C. fetus ssp. jejuni and C. coli, contrary to what has been reported by some (16, 44) seems to be useless. We found all our strains to be totally incapable of growth in media with 8% glucose. Also, they have no fermentative activity on 1% glucose, and both of these findings are in accordance with recent reports (10, 37).

A preliminary differentiation between C. fetus ssp. jejuni and C. coli can be made on the basis of 3 tests. Growth at 30.5° and at 45.5° C and hydrolysis of sodium hippurate can serve to distinguish between the 2 organisms (Table 4). But the specificity of these tests in distinguishing the 2 organisms seems to be complicated by appearance of strains with intermediate characteristics whose reactions suggest their relatedness to both distinct organisms. In view of these findings, it appears that C. fetus ssp. jejuni and C. coli are very closely related

Table 4. Main features and subgroup characteristics of *C. fetus* ssp. *jejuni*/*C. coli*

	jejuni strains							*coli* strains				Intermediate strains				
	Group 1							Group 2				Intermediate				
	Hipp.[a] + 30.5°C - 45.5°C -							Hipp. - 30.5°C + 45.5°C +				Hipp. - 30.5°C + 45.5°C -		Hipp. + 30.5°C - 45.5°C +		Hipp. - 30.5°C - 45.5°C +
Sub-Group	1 (G1)	2 (G1)	3 (G1)	4 (G1)	5 (G1)	6 (G1)	7 (G1)	1 (G2)	2 (G2)	5 (G2)	6 (G2)	1 (I)	4 (I)	5 (I)	7 (I)	5 (I)
Test																
4% TTC[b]	T[c]	S	T[c]	S	T	S	T	T	S	T	S	T	S	T	T	T
1% Glycine	+[c]	+	-[c]	-	+	+	-	+	+	+	-	+	-	+	-	+
DNase	+	+	-	-	-	-	+	+	+	-	-	+	-	-	+	-
	750	1350	958	890	756	746	652	440	1353	1143	1388	748	753	657	1269	1355
	764	1351	751	1145	767	747	1220		1440	1380				1211		
	1114		754		1334	778	1375			1207				1349		
	1299		755		1370	1369				1442						
	1441		776		1268	1373				426						
			917		1352	1374										
					1379	1385										
					1383	1386										
					1384	1443										
					1387											

[a] Hippurate hydrolysis.

[b] Triphenyl tetrazolium chloride; T = Tolerant; S = Sensitive.

[c] + = positive; - = negative.

to the extent that the presently available tests fail to separate them into 2 distinct species. In order to confirm or deny King's (27) assumption 3 decades ago that her "related vibrios," Vibrio coli and vibrios of avian hepatitis are identical to Vibrio jejuni, a study of genetic relationship such as nucleic acid homology seems to be essential. For the purpose of recognizing subgroups within Group 1 (C. fetus ssp. jejuni) and Group 2 (C. coli) 3 biochemical tests, "TTC (Fig. 3), glycine, and DNase production (Fig. 4)" gave significant results. C. fetus ssp. jejuni strains (Group 1) were placed in 7 subgroups (1 through 7) with 5 strains in subgroup 1, 2 in subgroup 2, 6 in subgroup 3, 2 in subgroup 4, 10 in subgroup 5, 9 in subgroup 6, and 3 in subgroup 7. C. coli strains (Group 2) were placed in 4 subgroups 1, 2, 5 and 6. These subgroups respectively have 1, 2, 5 and 1 strains of C. coli. Four of the strains with intermediate characteristics fell in the most prevalent subgroup 5 and one strain in each of subgroups 1, 4 and 7. These are most likely atypical strains of C. fetus ssp. jejuni and C. coli. Owing to the small number of strains studied and the necessity of establishing the definitive relationship between C. fetus ssp. jejuni and C. coli prior to establishing biotypes, we used the word subgroups in place of biotypes. We hope that this provides a basis for further studies that can clarify the close relationship that exists between these organisms.

Fig. 3. Sensitivity to triphenyl tetrazolium chloride

A. Campylobacter fetus ssp. fetus strain (482) sensitive strain

B. Campylobacter fetus ssp. jejuni strain (958) tolerant strain

C. Campylobacter fetus ssp. jejuni strain (1369) sensitive strain

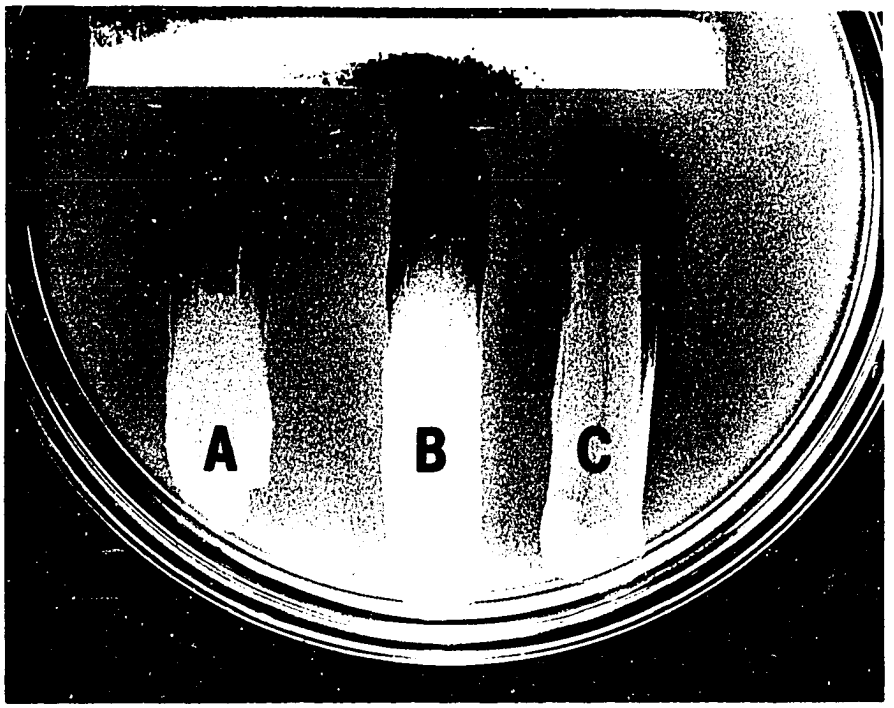
Dark area at the tip of growth shows where the diffused TTC has been reduced to red formazan by the growing organisms

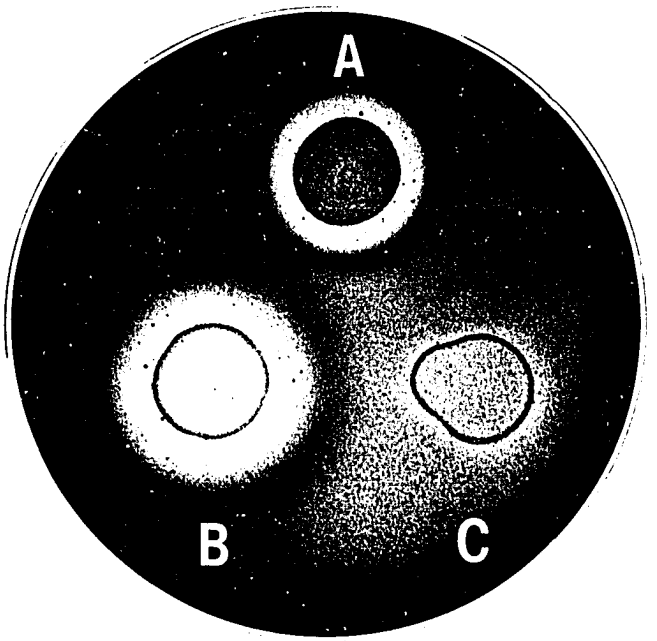
Fig. 4. DNase production

A. Serratia marcescens; the positive control bacterium

B. Campylobacter fetus ssp. jejuni strain (652) positive reaction

C. Campylobacter fetus ssp. jejuni strain (958) negative reaction


A
B
C


A
B
C

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SEROTYPING *CAMPYLOBACTER FETUS* SUBSPECIES *JEJUNI*/ *CAMPYLOBACTER COLI* FROM MAN AND ANIMALS

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Abstract

Sixty-one test strains of Campylobacter fetus ssp. jejuni and Campylobacter coli from human and six animal species were subjected to serotyping by the passive hemagglutination-microtiter plate technique. Antisera against live whole-cell antigens (LWC-Ag) from 34 Penner reference strains designated T1 through T39 were used for the serotyping. Cultures of the test strains were harvested, steamed (ST-Ag) and then used to sensitize sheep red blood cells for the titration of the typing antisera. Antisera against autoclaved test strains (AU-O-Ag) were also titrated with the ST-Ag-sensitized SRBC. Forty-five of the test strains were found to be typeable by antisera against 17 Penner serotype strains. All the ovine strains were found to be typeable with the majority (71%) reacting equally to serotypes T1 and T8. The human strains were equally distributed among 7 different serotypes. Fifty percent of the canine strains could not be placed in any of the available Penner serotypes. The majority of the strains reacting to T8 antiserum were of ovine origin. Serotype T2, the most common, was found among the strains isolated from a majority of the hosts represented but did not predominate in any host. Serotype T1 was the second most common. Serotypes T2 and T4 were encountered with equal frequency among the bovine strains. Isolates of C. fetus ssp. jejuni and C. coli can be typed by the passive hemagglutination technique. The antisera against the autoclaved antigens did not react with the sensitized SRBC.

Introduction

Campylobacter fetus ssp. jejuni is a microaerophilic bacterium that causes abortion in sheep (5, 9, 18). It has been incriminated in enteritis, diarrhea and dysentery in animals (1, 12, 13) and associated with gastroenteritis and other conditions in man (4, 16). Campylobacter coli considered by some (6, 8) as one of the etiological agents of swine dysentery although this has never been well substantiated. Both organisms have been isolated from sick and healthy animals and man (4, 7, 19). They have several biochemical characteristics in common and on biotyping attempts we found them (manuscript 1 of this dissertation) as others did (14, 17) to be very closely related. As a result they are now being referred to as the "jejuni/coli group" (17, 20). Since biotyping has failed to distinctly separate these organisms, it was thought that serotyping might reveal differences which could be useful in determining the interhost relationships of the jejuni/coli group.

Serotyping of these organisms is necessary for understanding the epidemiology of the infections they produce in man and animals. But the most widely used method for serotyping of bacteria (Slide and tube agglutination procedure) is not useful for testing this group of organisms because of the autoagglutinating property of the jejuni/coli group (3). Modified and improved serotyping techniques recently have been developed in order to adapt them for typing isolates of C. fetus ssp. jejuni (11, 15). The most promising approach so far is the typing by

the hemagglutination technique of Penner and Hennessy (15) and of Dr. Lauwers of Free University, Brussels, Belgium (personal communication).

Twenty-three basic serotypes of C. fetus ssp. jejuni have been identified (15) which can be used to type other isolates of this organism. Up to 30 additional serotypes have been identified since then by Dr. Penner of the University of Toronto, Canada (personal communication).

The purpose of this investigation was to serotype 61 Campylobacter isolates from clinical and nonclinical infections in cattle, sheep, pigs, dogs, chickens, monkeys and humans in order to determine their antigenic relationship to the Penner serotype strains.

Materials and Methods

Test strains. Forty-four Campylobacter fetus ssp. jejuni, 13 Campylobacter coli together referred to as the jejuni/coli group, and 2 of each of C. fetus ssp. fetus and C. fetus ssp. intestinalis were used in this study. The strains of the latter 2 subspecies were included for comparison. The identity of these strains was established by a previous cultural and biochemical study (manuscript 1 of this dissertation) that utilized several differential tests, the most important of which were growth at 25° and 43° C, sensitivity to naladixic acid and cephalothin, tolerance to 4% triphenyl tetrazolium chloride, H_2S production and hydrolysis of sodium hippurate. All the strains were obtained from a stock culture collection maintained by the second author at the National Animal Disease Center (NADC) at Ames, Iowa. Their designated laboratory numbers, origins and identifications are presented in Table 2.

Penner serotype strains. Thirty-four C. fetus ssp. jejuni of human and animal origin were obtained from the Centers for Disease Control (CDC), Atlanta, Georgia. They were used as reference serotyping strains. These were originally provided by Dr. Penner of the University of Toronto, Canada, who typed them by the passive hemagglutination technique into 34 serotypes designated T1 through T39 with the exception of T6, T12, T14, T23 and T29. Antisera against the Penner serotype strains were used to type the Campylobacter strains used in this study.

Preparation of autoclaved antigen (AU-O-Ag) from test strains. Test strains were streaked on the surface of Brucella agar (BrA) plates prepared from Brucella agar Driform (Gibco Diagnostics, Madison, Wisconsin) as directed by the manufacturer. The plates were incubated microaerophilically[a] for 48 h at 37° C. Purity of growth and homogenicity of cell morphology were examined. A single colony was subcultured on BrA plate and incubated as above for 24 h. Then 4 BrA plates were subcultured from the resultant growth and incubated as above. After 24 h, the confluent growth on the 4 plates was harvested into a 250 ml flask containing 100 ml Brucella broth (BB) prepared from Brucella Broth (Albimi) Dri-Form (Gibco Diagnostics) as directed by the manufacturer. Flasks were incubated at 37° C statically for 1 h and then in a gyratory water-bath shaker (130 agitations/min) for 5 h. The broth culture was centrifuged in a refrigerated centrifuge at 4,080 g for 30 min. The supernatant was discarded and the pellet was resuspended

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[a]In desiccators containing 5% O_2, 10% CO_2 and 85% N_2.

in 10 ml distilled H_2O and autoclaved for 2 h at 121° C. Forty ml of distilled H_2O was added to the autoclaved cell suspension, thoroughly mixed and centrifuged at 3020 g for 30 min. This step was repeated twice and the final pellet was suspended in 5 ml saline and stored at 4° C as an autoclaved somatic antigen (AU-O-Ag).

Standardization of AU-O-Ag and production of antisera in rabbits. A portion of the AU-O-Ag suspension was diluted with 0.85% NaCL solution in 13 x 100 mm screw cap tubes and standardized to 40% T in a spectrophotometer (Spectronic 20 Model 340 Bausch and Lomb) at 540 nm and then stored at 4° C. Five to seven lb female New Zealand rabbits were prebled and then injected intravenously with the standardized AU-O-Ag. Protocol of immunization was similar to that of Berg et al. (2). Rabbits were exsanguinated 7 days after the last injection was given. The sera were separated, put into 4 dram vials and 1% percent merthiolate was added at a rate of 0.1 ml/10 ml serum and stored at 4° C until used.

Preparation of live whole-cell antigen (LWC-Ag) from Penner serotype strains. Each Penner reference strain was streaked on Bacto-Mueller-Hinton medium prepared as described by the manufacturer (Difco Laboratories, Detroit, Michigan). Cultures were incubated microaerophilically at 37° C for 24 h. Single colonies were subcultured and incubated as above for 18 to 24 h. The growth was harvested into 0.85% NaCl solution and the density of the suspension was standardized to McFarland tube No. 10. Two ml of the standardized suspension was thoroughly mixed with 2 ml of Freund's complete adjuvant in a vial for rabbit immunization.

Production of typing antisera against LWC-Ag of Penner strains. Rabbits were injected subcutaneously in the abdominal area and the thigh with 1.5 ml of the LWC-Ag (0.3ml/injection site) 2 injections in the upper abdominal part, one injection in the middle abdomen and one injection into each of the two thigh muscles. A week after the primary injection, 0.5 ml ephedrine was injected into the thigh muscle. Thirty min later, 0.5 ml live bacterial suspension equal to density of McFarland No. 10 was injected into the ear vein. The injection of the live bacterial suspensions was repeated 3X at an interval of 3 days apart. Seven days after the last injection the rabbits were bled through cardiac puncture and antisera were separated, preserved and stored as described above.

Preparation of steamed antigen (ST-Ag) from test strains. Each strain was grown on BrA plate as before for 24 h. The growth was harvested into 0.85% NaCl solution, washed once, resuspended in 5 to 7 ml saline, and steamed in an autoclave at 100° C (low pressure) for 2 h. The steamed suspension was further diluted with saline to a density equal to that of McFarland tube No. 9 and stored at 4° C until used. Each ST-Ag from a test strain at this density was used to sensitize the sheep red blood cells (SRBC) which were employed in the titration of the various antisera.

Sensitization of sheep red blood (SRBC) with ST-Ag. The procedure followed here is that of Dr. Lauwers (personal communication). Thirty ml of sheep blood was collected into 30 ml of Alsever's solution (10) and stored at 4° C until used. Fifteen ml of the SRBC mixture was

washed 5 X with an equal volume of modified barbitol buffer (MBB) (10). One volume of a 10% SRBC suspension was added to an equal volume of ST-Ag, incubated for 2 h at 37° C, centrifuged and the supernatant was discarded. The packed cells were washed 3X with MBB and finally suspended to 0.5% sensitized SRBC suspension. A suspension of nonsensitized SRBC at a similar concentration was also prepared.

Hemagglutination scheme in microtiter plates. Microtitration multi-well plates (Flow Laboratories, Hamden, Connecticut), two sets of pipette droppers (Dynatech Laboratories, Alexandria, Virginia) .025 and .05 ml/drop capacity and microdiluters (Cooke Engineering Co., Alexandria, Virginia) were used to perform the hemagglutination test.

Titration of antisera. The protocol for titrating the antisera is presented in Table 1. Microtiter plates were covered with adhesive plastic tape and incubated at 37° C for 1 h, refrigerated at 4° C for 18 h and read by the help of a concave microtiter mirror under direct overhead light. Results were recorded positive reaction for a shield and negative reaction for a button. If heteroantibody was detected, absorption of the antiserum with nonsensitized SRBC was carried out and retested.

Controls. Well no. 1 of every row served for heteroantibody detection. Well no. 11, row A, served for sensitized SRBC autoagglutination detection. Well no. 12, row A, served for nonsensitized SRBC autoagglutination detection.

Table 1. Protocol for hemagglutination test in microtiter plate[a]

Serum Dilution	1/5	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280		
Well #	1	2	3	4	5	6	7	8	9	10	11	12
Diluent MBB(µl)[b]			25	25	25	25	25	25	25	25	25	25
Antiserum (µl)	25	25	25	—	—	—	—	—	—	—		(25µl) discard
MBB (µl)	50	50	50	50	50	50	50	50	50	50	50	50
Nonsensitized SRBC[c] (µl)	50											50
Sensitized SRBC(µl)		50	50	50	50	50	50	50	50	50	50	50

[a]This represents row A of the microtiter plate. Rows B-H were similarly treated except tests of wells 11 and 12 were not needed here.

[b]MBB = Modified barbital buffer.

[c]SRBC = Sheep red blood cells.

Results

Forty-five of the 61 strains (74%) were placed into 17 of the 34 Penner serotypes. The majority of the ovine strains, a human, and an avian strain, reacted similarly with antisera against T1 and T8 in equal titers and could not be assigned to one single serotype. They were therefore assigned to a combination of the 2 serotypes (T1/T8). Eighteen of the typeable strains showed cross reactions with one or more of the serotypes. The antiserum titers for the principal serotypes ranged from 320 to 655,360 and the majority were above 1280. The cross-reacting titers ranged from 80 to 20,480. Sixteen strains including the 4 strains identified as subspecies fetus and subspecies intestinalis were found to be untypeable by the antisera to the 34 Penner serotype strains. Of the untypeable jejuni/coli strains two were from cattle, 2 from pigs, 2 from humans, 2 from chickens and 4 from dogs. The test strains from ovine origin were found to be typeable only by antisera against 2 of the Penner serotype strains (T1 and T8). None of the antisera produced against the autoclaved test strains (AU-O-Ag) reacted with the SRBC sensitized with the steamed antigens (ST-Ag). The basis for assignment of each test strain to a given serotype was its comparative reaction to various dilutions of each different typing serum. The designated serotypes and the reaction titers are given in Table 2.

Discussion

Hyperimmunization of rabbits with live whole cell antigens in Freund's complete adjuvant resulted in high titer antisera that was very

Table 2. Serotypes of Campylobacter strains tested and titers of heterologous antisera to Penner serotype strains

Strain designation[a]	Campylobacter identification	Hippurate reaction	Penner serotypes and reactions		
			Principal serotype	titer[b]	Crossreacting serotype
0-652	ssp. jejuni	+	T1,T8	1280	
0-657	" "	+	T1,T8	2560	
0-917	" "	+	T8	10240	T1
0-958	" "	+	T8	2560	T1
0-1114	" "	+	T1,T8	2560	
0-1220	" "	+	T1,T8	5120	
0-1269	" "	+	T1,T8	1280	
B-746	" "	+	T2	5120	
B-747	" "	+	T35	20480	
B-748	C. coli	-	T34	327680	T30,T26
B-750	ssp. jejuni	+	-		
B-751	" "	+	T4	640	
B-753	" "	+	T36	320	
B-754	" "	+	T2	10240	
B-755	" "	+	T3	320	
B-756	" "	+	T4	163840	T3,T13,T16
B-764	" "	+	T36	640	
B-767	" "	+	-		
B-776	" "	+	T19	10240	
B-778	" "	+	T4	1280	
B-890	" "	+	T2	5120	T1,T8
H-1207	C. coli	-	T34	81920	T26,T30
H-1211	ssp. jejuni	+	T36	20480	
H-1299	" "	+	-		
H-1334	" "	+	T37	655360	
H-1349	" "	+	T2	5120	T15
H-1369	" "	+	T13	1280	
H-1370	" "	+	-		
H-1460	" "	+	T3	20480	

H-1482	" "	+	T1,T8	320	
S-426	C. coli	-	-		
S-440	" "	-	T20	655360	
S-1143	" "	-	T24	327680	T26
S-1380	" "	-	T5	81920	T31
S-1486	" "	-	T24	1280	T26
S-1488	" "	-	-		
Bd-1145	ssp. jejuni	+	-		
C-1268	" "	+	T1,T8	2560	
C-1350	" "	+	-		
C-1351	" "	+	T13	2560	T16,T4,T3
C-1352	" "	+	T5	327680	T31
C-1353	C. coli	-	T20	81920	T34,T30,T26
C-1355	" "	-	T30	10240	T39
C-1373	ssp. jejuni	+	T2	2560	
C-1374	" "	+	T2	2560	
D-1375	" "	+	T16	40960	T13
D-1379	" "	+	T13	20480	T16,T3,T4
D-1383	" "	+	-		
D-1384	" "	+	-		
D-1385	" "	+	T2	10240	
D-1386	" "	+	T2	10240	
D-1387	" "	+	-		
D-1388	C. coli	-	-		
M-1440	" "	-	T28	163840	T26
M-1441	ssp. jejuni	+	T35	5120	
M-1442	C. coli	-	T28	131720	T30,T26
M-1443	ssp. jejuni	+	T3	640	
B-482	ssp. fetus	-	-		
B-1289	" "	-	-		
B-436	ssp. intestinalis	-	-		
B-1254	" "	-	-		

[a]The origin of each isolate is designated by a letter preceding the isolate number; (O = ovine, B = bovine, H = human, S = swine, Bd = bird, C = chicken, D = dog, M = monkey).

[b]Titers are reciprocal of dilutions; Titers of crossreacting antisera ranged from 80-20480.

specific and reactive in the passive hemagglutination test. The antisera were highly discriminatory between subspecies but relatively less so among members of the same subspecies. Several strains biochemically identified as C. fetus ssp. jejuni were of the same serotype as some strains identified as C. coli. This suggests that these two organisms share common antigens and some of them may be antigenically identical or very closely related. This strengthens the recent opinion (manuscript 1) that C. fetus ssp. jejuni and C. coli cannot be definitively differentiated only by biochemical and serological bases.

The most frequently occurring serotypes of the strains studied were T8 (27%), T2 (24%) and T1 (21%). Serotypes T1 and T8 are the only serotypes detected among the ovine strains. These 2 serotypes are not detected among the bovine strains. The antisera against T1 and T8 reacted equally with one human, one chicken and 5 of the ovine strains resulting in identical titers in each case. This reflects common antigenic components in the 2 immunizing Penner serotype strains as well as in the test strains that were found typeable into these 2 serotypes. Serotype T2 and T4 are more common among the bovine strains. Serotype T4 was not detected from the test strains of the other host species. Serotype T2 has a wider host range than any other of the detected serotypes. It suggests that the antigenic components of Penner serotype T2 are more common and present in strains isolated from the numerous host species. The bovine and the avian strains are relatively more widely distributed among the detected serotypes. They belonged to 7 and

6 serotypes, respectively. The ovine strains were 100% typeable into only 2 of the detected Penner serotypes. Only 50% of the canine isolates were typeable. The untypeable canine isolates may belong to the 3 other Penner serotypes which were not included in this study. Interestingly, the canine strains are the only strains from one geographical location (Denver, Colorado).

The finding that SRBC suspension sensitized with ST-Ag do not react with antisera against AU-O-Ag strongly suggests that the sensitizing antigenic components of the steamed Campylobacter cell suspension (ST-Ag) are different from the components of the autoclaved and washed suspension (AU-O-Ag). In a preliminary trial utilizing the agglutination test in microtiter plates "button test" we found the AU-O-Ags to react with their homologous antisera giving respectable titers as high as 8192 whereas reaction with the Penner type antisera using the passive hemagglutination technique the ST-Ags resulted in titers as high as 655360. However as a result of the numerous nonspecific cross reactions encountered between the test strains when the button test was used, we abandoned this test and proceeded with the passive hemagglutination technique which seemed more specific. It is evident that the antigenic components of the AU-O-Ag are chemically different from the components of the ST-Ag that has SRBC sensitizing capacity. It is quite possible that autoclaving destroyed certain antigenic components which were not affected by the steaming process. It is also possible that the Au-O-Ag contained only somatic antigen whereas the ST-Ag contained somatic,

flagellar and membrane surface components. It is evident that strains of the jejuni/coli group do not share cell surface antigens with subspecies fetus and subspecies intestinalis.

The results of this study indicate that the strains of C. fetus ssp. jejuni and C. coli can be typed by the passive hemagglutination technique. Sharing of common heat stable cell surface antigens by some strains of the two organisms is evident. Antisera against autoclaved cells of the jejuni/coli group do not react with SRBC sensitized with components of steamed cell suspension of the same group of organisms.

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EVALUATION OF THE PATHOGENICITY OF *CAMPYLOBACTER FETUS* SUBSPECIES *JEJUNI*/*CAMPYLOBACTER COLI* STRAINS IN THE PREGNANT GUINEA PIG MODEL

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Abstract

The pathogenicity of 17 Campylobacter strains for the pregnant guinea pig (G. pig) was investigated. Twelve of 14 (86%) of C. fetus ssp. jejuni/C. coli strains produced abortions ranging from 13% to 87%. Two strains did not produce any abortions. Reference strains of C. fetus ssp. fetus produced 60% to 87% abortion and C. fetus ssp intestinalis produced 60% abortion. Reisolation of inoculated organisms from uterus, blood, liver, kidney, spleen and gallbladder of inoculated G. pigs was accomplished at a rate as high as 83% from two ovine strains and as low as 13% from two bovine and one human strain. The majority of the reisolations were from the uterus. No reisolation of either of the two chicken strains was made. Within the jejuni/coli-group, the ovine and the human strains seemed to be more pathogenic. The swine, bovine and chicken strains were less pathogenic. The results strongly support the use of the pregnant G. pig model as a suitable and practical procedure for evaluating the pathogenicity of Campylobacter organisms regardless of their host of origin.

Introduction

Campylobacter fetus (formerly Vibrio fetus) has long been recognized as a causative agent of infertility and abortion in cattle and sheep (9, 27). Campylobacter fetus ssp. jejuni and Campylobacter coli were incriminated in intestinal disorders, dysentery and diarrhea in various animals and hepatitis in chickens although the evidence was not always concrete (2, 10, 14, 20). They have also been associated with enteritis and other infections in man (4, 15, 21, 24). However, within recent years several investigators have reported the isolation of C. fetus ssp. jejuni and C. coli from feces of healthy animals, chickens and other birds (1, 6, 18, 23, 25, 26, 27). Isolation from stools of asymptomatic human carriers was also reported (7, 8). The significance of finding these organisms in healthy animals and asymptomatic humans is not clear, and whether they are pathogenic, commensals or symbionts awaits further investigation. This situation stimulated our interest in evaluating the pathogenicity of Campylobacter isolates from different hosts by testing their effect on susceptible experimental animals. Earlier investigation of infectivity of C. fetus ssp. jejuni and C. coli for digestive organs of the orally inoculated domestic animals and nonhuman primates revealed unconclusive results (2, 3, 12, 13, 30). On the other hand, the pathogenicity of different campylobacters for pregnant cows and ewes has been proven (6, 11, 19), but their use is expensive and is not always practical on routine basis. Therefore, a simpler method of in vivo assay was proposed, such as the pregnant guinea pig (17, 22). Bryner et al. (5)

determined the minimum abortive dose for an ovine strain of C. fetus ssp. jejuni for the pregnant guinea pig and recommended the use of the pregnant guinea pig model to test efficacy of Campylobacter vaccines.

The purpose of this investigation was: (1) to determine the pathogenicity of 13 Campylobacter fetus ssp. jejuni/Campylobacter coli isolates from humans, cows, pigs, sheep and chickens by determining their abortifacient effect in the pregnant G. pig in comparison to 4 known pathogenic strains of C. fetus ssp. fetus, C. fetus ssp. intestinalis and C. fetus ssp. jejuni of bovine and ovine origin, (2) to substantiate the suitability of the pregnant G. pig model for testing the pathogenicity of Campylobacter isolates from sources other than the genital organs and aborted fetuses.

Materials and Methods

Pregnant guinea pigs. Two hundred ninety-five English short hair (Hartley strain) pregnant guinea pigs (G. pigs) approximately 6 months old were obtained from the closed colony breeding unit of the National Animal Disease Center at Ames, Iowa. The G. pigs were in their third to fourth week of pregnancy and weighed around 630 g. They were received in 4 batches; 55 in the first batch for experiment I, 70 in the second batch for experiment II, and 85 in each of the third and fourth batches for experiments III and IV. Shortly after delivery the G. pigs were palpated for pregnancy by the Matthews and Jackson's technique (16), then randomly divided into units of 5 in a cage and were allowed to rest

for 24-36 h. Each 3 units (15 G. pigs) constituted a group for testing 3 inoculum levels of one bacterial strain. In each experiment, 2 units (10 G. pigs) served as unchallenged controls for the experiment. With an electrical balance model Mettler P2010N, the G. pigs were weighed each morning until the day of sacrifice or end of experiment.

Campylobacter strains. Seventeen catalase positive Campylobacter strains of which 5 were from cattle, 2 from sheep, 5 from humans, 3 from pigs and 2 from chickens were selected for pathogenicity studies from a large number of stock cultures maintained under lyophilized or frozen conditions by the second author at the National Animal Disease Center at Ames, Iowa. Selection was based on 4 criteria: (1) recent isolate, (2) low passage, (3) good growth on blood agar plates and high viable cell counts, and (4) having typical Campylobacter cell morphology and motility. The laboratory number, source, serotype and identification of the selected strains are presented in Table 1.

Culture media and growth conditions. Blood agar plates (BA) were prepared by dissolving 52 g dehydrated Brain heart infusion agar (BBL, Cockeysville, Maryland, Catalog No. 11065) in a liter of water and autoclaving it at 121° C for 15 min. Then 10% bovine blood was aseptically added prior to pouring into petri plates.

Brucella broth. Fluid Brucella broth (BB) was prepared from Brucella Broth (Albimi) Dri-Form (Gibco Diagnostics, Madison, Wisconsin, catalog No. M08600) as directed by the manufacturer and dispensed into 250 ml flasks (100 ml/flask) or into 16 x 150 mm tubes (10ml/tube).

Fluid thioglycollate medium. 29.5 g dehydrated fluid thioglycollate medium USP (BBL, Catalog No. 11260) with 0.75 g agar was dissolved in a liter of H_2O by heating to a boil, autoclaved at 121° C for 15 min and dispensed into 18 x 15 mm tubes with Morton closures (10 ml/tube).

Preparation of challenge inocula and intraperitoneal inoculation of G. pigs. Frozen or lyophilized strains were activated on BA plates incubated at 37° C for 48 h under microaerophilic conditions (in desiccators containing 5% O_2, 10% CO_2 and 85% N_2). Purity of growth was ascertained and single pure colonies were subcultured on BA for 24 h. The growth was harvested into 10 ml broth and the optical density (OD) was adjusted with broth to 0.2 OD at 550 nm using spectrophotometer (Spectronic 20 Baush and Lomb). One ml of the suspension with 0.2 OD was transferred to a tube containing 9 ml of broth, then a 10-fold serial dilution was performed in a series of tubes each containing 9 ml of broth from tube no. 1 through tube no. 8. Three inoculum levels (high, medium and low) of each strain representing 10^{-1}, 10^{-5} and 10^{-8} dilutions of 0.2 OD were used for G. pig challenge. The challenge dose for each G. pig was one ml of the above concentrations. Immediately after the serial dilution each level of inoculum was injected intraperitoneally into 5 G. pigs (15 G. pigs/strain).

Counts of viable cells. Plate counts of viable cells were performed on the 10^{-1}, 10^{-5} and 10^{-8} bacterial suspensions used for G. pig challenge. 0.1 ml of each concentration was streaked on the surface of

BA plates (4 plates/concentration) and were incubated microaerophilically at 37^{o} C for 3 to 5 days. Also, 2 thioglycollate tubes were inoculated per concentration, each with 1 ml of the broth dilutions. Colonies appearing on the surface of the BA plates were counted by the aid of a dissection microscope at 20 X magnifcation and an average of viable cells (CFU/ml) per inoculum level was obtained for each strain. The viable cell counts for the high inoculum levels ranged from 2×10^{7} to 6×10^{8} CFU/ml. The range of viable cell counts for the medium and the low inoculum levels were 2×10^{3} to 6×10^{4} and 2×10^{0} to 6×10^{1} CFU/ml respectively. The overall averages of the viable cell counts for the 3 inoculum levels are as follows: High inoculum level had 1.4×10^{8} CFU/ml. Medium inoculum level had 1.4×10^{4} CFU/ml and low inoculum level had 1.4×10^{1} CFU/ml.

Identification and sacrifice of aborting G. pigs and cultural reisolation of organisms. Inoculated G. pigs were examined twice a day for signs of abortion such as presence of aborted fetuses in cage, noticeable vaginal bleeding or sudden substantial loss of weight. All aborted and suspect animals were sacrificed on the day of abortion by intramuscular injection of succinylcholine chloride. Samples of heart blood and gallbladder contents were separately cultured into thioglycollate tubes. Placenta and/or uterine tissues and organ samples of liver, kidney and spleen were collected from each G. pig. The uterine tissues and the organ samples were homogenized separately in blenders and each was cultured into BA plates and thioglycollate tubes.

The plates were incubated microaerophilically at 37° C for 3 to 5 days. The tubes were incubated at 37° C in air for 7 to 10 days. The plates were examined under a dissection microscope at 20 X magnification for Campylobacter colonies and smears of suspect colonies were examined under a phase contrast microscope to confirm identity of Campylobacter colonies. The thioglycollate tubes were visually examined for appearance of Campylobacter growth. Cultures and smears were viewed under phase-contrast microscope for presence of viable Campylobacter cells. Results of the plate cultures were compared with those of tube cultures. All nonaborting and control G. pigs were sacrificed at the end of 3 weeks and necropsied as per the above routine. All aborting and nonaborting G. pigs from which the challenge organisms were culturally reisolated were considered infected with the challenge organisms.

Results

Clinical observations. The majority of the inoculated G. pigs exhibited weight loss, signs of abortion such as vaginal bleeding or aborted. The overall average weight loss was 67 g/aborting G. pig. None of the uninoculated control G. pigs exhibited signs of abortion or weight loss.

Infection and abortion. Inoculated G. pigs aborted as early as 2 days and as late as 17 days postinoculation. Aborted fetuses were fresh (Fig. 1) and rarely appeared oedematous or decomposed. The highest abortion rate was 13/15 (87%), produced by two (a reference and a test) strains. The lowest was 1/15 (7%) where only a single abortion was

Fig. 1. Three fresh, nondecomposed fetuses aborted by a guinea pig injected with 1×10^4 viable cells of Campylobacter fetus ssp. jejuni, which was later reisolated from the uterus of the aborting guinea pig

produced by a test strain. Table 2 shows the rates and percentages of abortions produced by each strain.

Infection and abortion produced by each group of Campylobacter strains with common host species of origin are presented in Table 3. None of the control G. pigs was infected or aborted (Table 2).

Cultural reisolation of inoculated organisms. Campylobacter organisms were readily recovered from the uteri, visceral organs, blood and gallbladder of the majority of the aborting G. pigs. The highest recovery rates were from the uterine specimen cultures (Table 2). In 3 nonaborting G. pigs reisolation of organisms from uterus and organ homogenate at the time of sacrifice was accomplished indicating that the campylobacters have uterotropism and also can reside in visceral organs of the body. Details of the recovery rates are shown in Table 2. Streptococcal organisms were isolated from uterine cultures of 3 of the aborting G. pigs, but from none of which campylobacters were reisolated. No isolation of campylobacter or any other organism was made from any of the control G. pigs.

Discussion

The results of this study provided evidence that Campylobacter isolates from sources other than the genital tract and aborted fetuses have the ability to produce abortion as do many of the stool isolates. Therefore, this characteristic can be used to measure their pathogenicity since abortion is an all-or-none effect. It is easier to recognize and

Table 1. Campylobacter fetus/ssp. jejuni, ssp. fetus, ssp. intestinalis and Campylobacter coli strains used in this study

Strain Lab. No.	Source	Identification	Penner serotype
482[a]	Bull semen	ssp. fetus	-
1289[a]	Bovine uterus	" "	-
1254[a]	Bovine fetus	ssp. intestinalis	-
750	Bovine gallbladder	ssp. jejuni	-
751	" "	" "	T4
958[a]	Ovine fetus	" "	T8
657	Ovine uterus	" "	TI,T8
1299	Human stool	" "	-
1349	Human stool	" "	T2
1460	Human stool	" "	T3
1482	Human stool	" "	T1,T8
1207	Human blood	C. coli	T34
1380	Swine fetal stomach content	" "	T5
1486	Swine feces	" "	T24
1488	Swine feces	" "	-
1353	Chicken feces	" "	T20
1355	Chicken feces	" "	T30

[a]Pathogenic reference strains.

Table 2. Abortions produced by, and reisolations of Campylobacter fetus ssp. jejuni and Campylobacter coli inoculated into pregnant G. pigs

Strain Lab. No.	Abortions No/total[a]	(%)	Cultural Reisolations positive/total[a] Uterine Homogenate	Organ Homogenate	Blood	Gall bladder
482[b]	13/15	(87)	10/15	5/15	6/15	0/15
1289[b]	9/15	(60)	9/15	6/15	6/15	0/15
1258[b]	9/15	(60)	8/15	7/15	6/15	4/15
750	1/15	(07)	1/15	0/15	0/15	1/15
751	3/15	(20)	1/15	0/15	1/15	0/15
958[b]	11/15	(73)	10/15	10/15	3/15	9/15
657	13/15	(87)	10/15	4/15	4/15	1/15
1299	3/15	(20)	3/15	0/15	0/15	1/15
1349	6/15	(40)	6/15	2/15	4/15	4/15
1460	7/15	(47)	7/15	3/15	4/15	7/15
1482	8/15	(53)	6/15	4/15	4/15	6/15
1207	2/15	(13)	2/15	0/15	0/15	0/15
1380	8/15	(53)	5/15	2/15	4/15	2/15
1486	5/15	(33)	4/15	1/15	1/15	2/15
1488	2/15	(13)	2/15	0/15	0/15	0/15
1353	1[c]/15	(07)	0/15	0/15	0/15	0/15
1355	2[c]+1/15	(20)	0/15	0/15	0/15	0/15
--	0/40[d]	(00)	0/40	0/40	0/40	0/40

[a]Total number of G. pigs inoculated/strain or total number of samples cultured.

[b]Pathogenic reference strains.

[c]Streptococcus organisms were isolated from the 3 aborted G. pigs.

[d]40 uninoculated control G. pigs.

quantitate abortion in the experimental animal than other effects such as intestinal colonization or diarrhea which produced little reaction (3, 12, 29, 30). The intraperitoneal route of inoculation proved effective in producing infection and subsequent abortion in pregnant G. pigs. The majority (60-87%) of the G. pigs that received the reference strains aborted, while the rate of abortions definitively due to the test strains varied from 0 to 87%. All members of the 3 subspecies of C. fetus produced varying degrees of abortion, and reisolations were made from most of the aborted G. pigs. But only 4/6 of the C. coli strains produced abortions from which reisolations were made. Two strains of C. coli were not reisolated from the aborting G. pigs which yielded streptococci that might have been responsible for those abortions. This indicates that C. coli strains have less potential to produce abortion than the subspecies of C. fetus.

In the test strains and within members of each subspecies, the degree of pathogenicity seemed to be host of origin related (Table 3). Strains of C. fetus ssp. jejuni from ovine source produced the highest rate of abortion (87%). Those from human sources resulted in up to 40% abortion rate. The bovine strains were the least abortigenic with an abortion rate of only 13%. Strains of C. coli were generally less abortigenic than strains of C. fetus ssp. jejuni. Those from swine were more abortigenic than those from chickens. The former produced 33% abortion rate while the latter resulted in only 13%. This does not imply that chicken strains are generally nonpathogenic because there is

Table 3. Infections and abortions produced in pregnant G. pigs by each group of the Campylobacter strains originating from a common host species

Host	Number of Strains Tested	Campylobacter identity	G. pigs aborted No./total	(%)	G. pigs infected No./total	(%)
Bovine	2	ssp. jejuni	4/30	(13)	4/30	(13)
Ovine	2	"	13/15, 11/15	(87),73	25/30	(83)
Human	4	"	24/60	(40)	24/60	(40)
Swine	3	C. coli	15/45	(33)	15/45	(33)
Chickens	2	"	4/30	(13)	0/30	(00)
Human	1	"	2/15	(13)	2/15	(13)
Bovine	2	ssp. fetus	22/30	(73)	24/30	(80)
Bovine	1	ssp. intestinalis	9/15	(60)	9/15	(60)

insufficient data on which to base such conclusion since only 2 strains of chicken origin have been studied, but in this study strains from other host species seemed more abortigenic.

Comparing the percentage of abortions produced by each of the 3 inoculum levels/ individual strain, it appears that the effect is dose dependent. This is because in all cases the earliest abortions and the highest incidences of abortion were produced by the largest number of organisms inoculated. But this was not always the case when we compare abortions produced by similar inoculum levels of 2 different test strains. Human strains 1482 and 1460 (C. fetus ssp. jejuni) had viable cell counts comparably below those of swine strain 1488 (C. coli) and chicken strain 1355 (C. coli). Nevertheless, the abortion rates of the 2 human strains were much higher (53%, 47%) than those of the swine and chicken strains (13%, 20%). The rate of abortion produced seemed to be affected by 3 factors which are: (1) the species of the host of origin, (2) number of viable cells in the inoculum, and (3) the identity of species or subspecies of the strain. All of the strains studied except the 2 chicken strains were capable of residing in the uterus of the G. pig and they were reisolated from the uterus more frequently than from any other organ. In one case, infection persisted up to the 4th week with no abortion and Campylobacter organisms were reisolated from the uterus of the G. pigs when destroyed. Early reisolation from blood and early and late reisolation from gallbladder and liver, kidney and spleen homogenate confirmed their dissemination through transient bacteremia and residence in these organs. The results of this study provide

evidence to confirm earlier observations (5, 22) that Campylobacter organisms are capable of producing abortion in the pregnant G. pig. Our results also substantiate the report (5) that the pregnant G. pig can be of practical use in measuring pathogenicity of Campylobacter organisms.

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GENERAL DISCUSSION AND CONCLUSIONS

The number of Campylobacter strains that have been isolated from humans and various animals within the last 3 years exceeds the total number of isolations encountered during the entire period since campylobacters were first discovered from animals. Part of the reason is the recent discovery that these organisms are of no lesser importance than Salmonella and Shigella as human enteric pathogens, a fact not known before. Nonetheless, it is rather an impediment to find that characterization and classification could not keep pace with the speed of isolation. As a result, a large number of recent isolates that are poorly characterized is being accumulated. Also the literature is providing a sizable body of incomplete information about these new isolates that seems to create more confusion in terms of classification and the taxonomic position of Campylobacters in general. Although the new isolation techniques (23, 68) have facilitated recovery of C. fetus ssp. jejuni/C. coli from stools, characterization and typing methods have not been perfected. The use of dual nomenclature continues to confuse many microbiologists and hinders effective exchange of information about these organisms. Scientists involved in Campylobacter research throughout the world should agree to adopt a nomenclature that eliminates triple naming, and unify the two existing nomenclatures.

The results of the studies pertaining to the cultural and biochemical characteristics of Campylobacter fetus ssp. jejuni/C. coli group clearly support the opinion that these organisms as a group are quite distinct

from the other 2 subspecies of C. fetus and from other catalase positive campylobacters as well. Tentatively they can be identified on the basis of their growth tendencies and morphological differences. Physiological characteristics such as growth temperature, antibiotic sensitivity and response to biochemical tests are adequate to distinguish them from the other 2 subspecies of C. fetus. The usefulness of some of the biochemical tests used earlier to distinguish between the subspecies of C. fetus seems to require reevaluation as I pointed out earlier in the results of the biochemical studies.

Preliminary differentiation between C. fetus ssp. jejuni and C. coli can be established on the basis of the hippurate reaction with some consideration given to the origin of the strain, although the latter is of a lesser importance. It appears from the findings of this investigation that C. fetus ssp. jejuni and C. coli are very closely related, and specific differentiation of every C. fetus ssp. jejuni strain from every C. coli is still a problem. It seems that some new and more definitive tests have to be developed to accomplish that goal. This investigation also provides a basis for subgrouping members of the C. fetus ssp. jejuni/C. coli group.

Serological analysis also supports the close relationship between some C. fetus ssp. jejuni and C. coli strains. Antigenic relationship between the two organisms was established by the serotyping. Several strains from both organisms fell into the same serotypes. This might further complicate classification efforts, but adequate serotyping is necessary in drawing inferences of epidemiological importance.

Establishment of the pathogenicity of new strains and the development of an animal model in which pathogenic strains can be studied is of no lesser importance than identification and classification. The animal models available today are not completely satisfactory. The pregnant G. pig model seems quite suitable for testing pathogenicity of strains for the gravid uterus regardless of their original site of isolation. However, to test the potential of members of the C. fetus ssp. jejuni/C. coli group to produce clinical enteric disease in humans and animals, will require the development of new animal models.

Conclusions derived from these studies are: (1) The members of Campylobacter fetus ssp. jejuni/C. coli group can be definitively identified from those of the other 2 subspecies of C. fetus by the biochemical techniques now available. (2) Campylobacter fetus ssp. jejuni and C. coli are very closely related and the present available tests are not adquate to separate them into two distinct species. (3) Tentative division of members of C. fetus ssp. jejuni/C. coli into 2 groups is possible by the aid of 3 biochemical tests. (4) Further subdivision of members of each group into subgroups is achieved for the first time with the help of 3 biochemical tests. (5) Antigenic relationship exists between members of C. fetus ssp. jejuni and C. coli but not between members of this group and those of subspecies fetus or subspecies intestinalis. (6) The members of the C. fetus ssp. jejuni/C. coli group are abortifacient pathogens in pregnant G. pigs.

The findings of these investigations answered some questions but raised others. (1) What new definitive tests are required to distinguish

between strains of C. fetus ssp. jejuni and C. coli and further establish permanent biotypes for each organism? (2) Are C. fetus ssp. jejuni and C. coli genetically similar and do they have the same percent guanine + cytosine ratio or do they constitute separate genotype groups? (3) What serological relationship exists between the strains of human origin and those from sources implicated as reservoirs for campylobacters causing gastroenteritis in humans? (4) What is the most appropriate animal model in which clinical signs of enteritis (diarrhea) can be produced? (5) What are the real pathogenetic mechanisms that are involved in campylobacter enteritis? These and other questions need to be answered by further investigations.

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